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"It is characteristic of Science and Progress that they continually open new fields to our visions."—PASTEUR.

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SOME QUESTIONS OF METHOD IN BACTERIOLOGY

E. G. HASTINGS[1]

University of Wisconsin, Madison, Wisconsin

The students at the University of Wisconsin have a form of salutation with which they greet both the home people and those from afield. To the latter it is often a cause of consternation for the salutation begins with a long, shrill hiss. You can well imagine the thoughts that rush through the mind of some dignified intellectual who has, with due formality, been introduced by the president of the university, or by some lesser light, and who steps forward to greet his audience, to be met with the first phase of this salutation. The salutation passes rapidly into the other phases which are clear in meaning and so the embarrassment is momentary. If the speaker has the resilience he should have to address a student audience, he recovers and smiles his thanks for their welcome.

I preface the sentence I am about to utter with these remarks in order that you will not judge it too hastily. Someone has said, "It is impossible to under estimate the ignorance of an audience." I am sure this was not said by anyone who was to speak to a group of his own faith, for each of his hearers would undoubtedly know as much and probably more of all the common phases of their branch of knowledge as would the speaker, unless it be about some narrow thing to which he may have devoted some special study and about which whatever he may say is likely to prove neither instructive nor entertaining. Groups of words with neither of these qualifications are best left unuttered. I find myself with an audience more versed than I in all the general phases of bacteriology and certainly not much interested in the few narrow fields to which I have devoted some

[1] President's address, Society of American Bacteriologists, December 29, 1923.

attention. And so the selection of a subject about which I might speak to you has been a difficult task.

It seemed worth while to present a few rather disjointed questions about some of the methods through the use of which we are attempting to extend our knowledge and to make more simple some of the things which appear so complex in the bacteriological world, for when the truth is found, it is apt to be simple and easy of comprehension. It is man's surmisings that fill our books with theories concerning the relations of things to each other, theories so intricate and involved that few can follow and none apply. Or again, we are using methods in control work of various kinds, and unless the methods are based on the truth, they cannot be of greatest value to us, indeed they may result in loss of time, energy, and money in doing things that are of no value in reaching our goal.

There are those who seem to think finality has been attained in some fields of study by our present methods of investigation. I quote from one in charge of the health activities of a great state, "The book of our knowledge of diphtheria, its cause, prevention and cure is closed for all time. Nothing remains to be known," or again from one in an allied field (and here I have left out the identifying adjective), "The tree of knowledge appears, however, to have grown to proportions which reveal the general outlines which it will always present. Further researches by the *methods* which have hitherto been so productive can, it seems, only clothe it in more attractive foliage and aid it in maturing the rich setting of fruit which has not ripened and fallen for the service of man."

I trust I do neither of these gentlemen an injustice when I interpret their remarks as indicating that they feel the methods which have been used in the study of the particular problems in which they are interested have revealed the truth, and nothing but the truth.

It seems to me that one who can make such a statement is very happy and contented. He is certain the ideal methods have been used. The results are final. They form the solid foundation on which others can build with assurance.

It also seems to me that it is a frame of mind not conducive to progress. Almost certainly there is a lack somewhere; and the results do not reflect truly the relations which exist between the myriad of associated living things and the effect of various agencies on them. An approach from a different angle may show the present knowledge to be a grotesque and distorted image of the truth. I think but few bacteriologists would consider any one of the methods they employ beyond improvement, and so may be willing to listen to a few thoughts on the tools rather than on the things made therewith.

A bacteriologist should run true to the form of the organisms which are the object of his study and interest. Possibly this is a cryptic sentence and needs explanation. In the course which I present each year to a group of agricultural students, I attempt to picture for them things as they are or rather as we think they are. I attempt to show that one great group of living things, the green plant, is doing one type of work in the world, the storing of the energy it receives in radiant form. From this store the remainder of the world of living things is to secure the needed supply. All other life than the green plant is dissipating energy, and gradually returning the elements of the food to the simple compounds which the green plant finds adapted to its needs. Among these destructive factors are to be found the bacteria; and hence, if a bacteriologist is running true to the form of his favorites, he will be destroying something, and that will probably be the tendency of what I may say to you tonight.

Viewed from another angle may not the work of the non-chlorophyl-bearing plants and of the animals in nature's scheme of affairs be as truly constructive as that of the green plants? Certain it is that it is as necessary, a fact I find very difficult of comprehension by my students after they have been told that the green plant makes its own food, and from this statement have gained the idea that the green plant lives unto itself alone, a completely independent existence whose continuity depends only on the radiant energy. If destruction is necessary for continued progress, or for the erection of something more useful, more true than the old, then surely it is not to be less considered.

A student using a modern text in bacteriology cannot but gain the impression that each of the various chemical transformations occasioned by bacteria is due to a specific organism possessing very definite characteristics which he can determine for himself in the laboratory, and having once learned these characteristics, he is in position to determine whether any other organism is identical with the first. The bestowal of a specific name, and the form of description leads him to think of a very definite cause. He goes into the bacteriological world and begins the study of a problem. He isolates, studies and names what he believes is a new organism. It may well have been an organism studied by a host of previous workers, and may in the future be studied by a host of others, and each be led to think he is the only one that has had the organism before his eyes. The thing that leads to this is the emphasis we place on most minute differences in morphology and function. We measure bacteria, we determine their ability to use this or that source of carbon, and of nitrogen, or we determine their limiting hydrogen ion concentrations, and practically all are done under such conditions that the results have little significance. We grow the organism in this and that and attempt to describe the manner of growth, its cultural characteristics, in minute detail.

It seems to me that we have expended and are still expending much energy which could better be used in other directions when we attempt to draw too fine lines of differentiation in our study of bacteria.

To my mind we are interested in processes, in results, and these processes will be carried on by a group of organisms related in morphology and in general physiology, and yet so differentiated that we must consider them as a group, and study them as a group. The morphology and physiology will be so varied that most general statements will suffice for the characterization of the group. We have gone some distance in the study of groups, but it seems to me we can abandon much of our detailed study of specific cultures and extend our study of groups, a point which I hope to make more clear a little later.

We isolate pure cultures and study their capabilities in this and that direction with the idea of finding out the rôle of an organism in nature. To work with impure cultures is repellent to the bacteriologist. There are many things, however, which indicate that work with what have been called naturally pure cultures will portray more faithfully the rôle of the group than will a much greater amount of work with the individual members of the group. I am sure most of you have encountered instances in which a mixture of strains is far more effective than any one strain alone. One certainly meets this in industrial fermentations.

As soon as we begin to work with mixtures of strains, our detailed specifications become general ones, much to the sorrow of the typical scientist who by tradition does things in the most detailed and correct manner, irrespective of the nature of the objects with which he is working or of the information he wishes to secure.

From mixtures of strains we may pass to mixtures of kinds, and reach a field the bravest hesitates to invade, and yet a field which must be explored if we are ever to find the truth of the rôle an organism plays in nature. Here again we must depart still more from our standards of morphology and physiology for an organism growing with another may vary widely from the characteristics shown when growing alone. Two kinds of acid-forming organisms may produce a higher acidity than either alone, the limiting hydrogen-ion concentration has changed. An organism growing in association with another may produce far more of a certain product, even though the associated organism has no power to form the product in question.

In nature the organisms are leading a complex social life. The associations are not hit and miss, but definite. The work of Dr. Hammer of the Iowa Agricultural College on the organisms associated with the Streptococcus lacticus group in the souring of milk and in the production of volatile acids and of substances which will impart flavor to butter is an example of definiteness in association. For years the dairy bacteriologists have taught that the practical butter maker should exert every effort to

keep his cultures of the lactic organism pure. They soon became contaminated with the associated organism, a condition which was essential to success in the practical use of the fermentation in butter making. We did our best in our ignorance to spoil the starters of the butter makers, but nature corrected our mistakes.

The average bacteriologist has been trained to have great faith in his microscopic examination of cultures. As a student he was led to believe that he could identify organisms from their microscopic appearance. Indeed in some courses practical examinations are given in which the student examines a set of slides and tells what organisms are before him. It may well be that he is able to identify a particular preparation as having been made from a culture of one of the organisms studied in the course, another from another and so on. I had the curiosity to go over many of the papers published during the last few years on anthrax in man and was surprised to find with what offhandness identification of the anthrax bacillus is made. A suspicious lesion, a stained preparation, a glance, and the thing is done. Some take a confirmatory step, the preparation of cultures, and a still smaller number the final part in settling the question through animal inoculation.

In glancing through the statistics of disease occurrence and of deaths, I was struck by the fact that there was the most varying ratio between cases of human anthrax and deaths from this disease. I wondered again if it did not mean faulty diagnosis because of false faith in methods.

It is true that in a certain environment an organism of a certain morphology may indicate the presence of the suspected one with some degree of certainty, but what magnitude the degree attains is always questionable and can be determined only by many confirmatory trials.

Last year I was indirectly in contact with the only industrially important bacterial fermentation other than the lactic and acetic acid industries. The process was easily interfered with by contaminating organisms. The first step in the control of the process was naturally to make a microscopic examination

of the unsuccessful fermentations. An organism quite different in morphology from the one used in the process was revealed. Henceforth all efforts in the line of control were directed to the study and elimination of the discovered organism. If abnormal fermentations were encountered in which the contaminating organism could not be found, they were explained in the most bizarre way. The faith of the men in charge of the control work in their methods was so great as absolutely to prevent them from thinking there might be another contaminating organism present whose presence was not revealed by any of their methods. A high degree of control was finally reached by using what I may call bacteriological sense. The unexplainable occurrences became simple when another contaminating organism was found. It indicated to me that one is safer in hanging on to fundamental biological ideas, and seeking for the source of trouble in the limitations of his methods rather than to place implicit faith in methods and to think that some condition has completely changed the physiology of the organism employed.

The detection of acid-fast organisms in the tissues and in sputa is generally looked upon as evidence of tuberculosis, neglecting such diseases as leprosy in man and Johne's disease in cattle.

I have wondered how often one is led astray by this method. How often in the history of bacteriology has the evidence revealed by the microscope been confirmed by animal inoculation? I suppose many times. But how often have positive microsopic results and negative results on inoculation been followed up? I presume in few if any cases. The tendency is to neglect such negative results, although the investigation of them may prove most fruitful in furthering our knowledge.

The use of Petroff's medium has sometimes resulted in the isolation of acid-fast organisms from sputum which were shown to be other than the tubercle bacillus. The literature contains considerable evidence that not all acid-fast bacilli in sputa are tubercle bacilli.

In the testing of cattle for tuberculosis, typical reactions are obtained in animals that show no lesions other than those of the

skin. Last year 3 per cent of the reacting cattle slaughtered in connection with the coöperative work of the federal government and the several states showed such lesions.

During the past year Doctor Beach and myself have examined a considerable number of such lesions. We have not been able to demonstrate the tubercle bacillus in a single instance. We have found in some cases acid-fast bacilli in the microscopic examination. We have been unable to culture them or to infect experimental animals with them. It would thus seem that the microscopic method of detecting tubercle bacilli has some error in directions not heretofore recognized, and that again there may be a group of acid-fast organisms that sensitize an animal to tuberculin.

The information supplied by the tuberculin test is not as definite and complete as those who are using it suppose. We need some new methods which shall enable us to unravel the relations of the acid-fast group.

There are other phases of the work with bovine tuberculosis in which the methods employed do not seem to be above suspicion. You are all aware of the general ideas held by the bacteriologist and the sanitarian concerning the relation of bovine tuberculosis to the disease in man. I presume you are not all informed concerning the magnitude of the work which is now being done to decrease the prevalence of bovine tuberculosis, since the reports of this work, as it is carried out by the federal government and the coöperating states, do not reach the journals upon which most of us rely for our information.

I presume a still larger part is not aware of the great amount of public money that is being spent in the struggle against this disease in cattle.

The work of attempting to limit the spread of bovine tuberculosis began in this country soon after tuberculin was found to be available as a diagnostic agent. Since 1893 the work has been constant in such important dairy states as New York and Wisconsin. It has been gaining momentum until within the past few years it has attained enormous proportions in some states, and has spread to all the states, many of which

in earlier years did nothing in regard to the disease, either in the way of reducing its occurrence within their borders, or of preventing the introduction from without.

States undertook programs that were doomed to failure because of the limitations of the methods on which the programs were based. The programs have been based on the belief in the absolute correctness, the infallibility of the tuberculin test, the belief that every animal which reacted to the test was tuberculous, and every animal which did not react was free from the disease. This belief was held by the great majority of the people who were concerned in the work, both by physicians and veterinarians, and indeed is still held to such an extent that it may endanger the ultimate success of the work of eradication of bovine tuberculosis. Of this more a little later.

Through the cumulative effect of many factors the general interest in this disease has become more wide spread and more intense. Indeed, the intensity is such in some quarters as to endanger the orderly progress of the movement to limit this disease. This intensity of interest is found in the trained sanitarian, the physician, the veterinarian and the farmer.

A recent and especially popular part of the tuberculosis eradication work is the area test. It consists in the testing of all cattle in an area, a county, the removal and slaughter of all reactors and the retesting of infected herds within a short time and the retest of all cattle in the area within a period of one to three years.

The work can be done very cheaply and rapidly when the intradermic test is employed. This test is probably as correct as the more time consuming and expensive thermal test. A man working under fairly favorable conditions can test a hundred animals per day, and the cost may not exceed 15 cents per animal.

This work has spread by leaps and bounds. It has a strong appeal to the practical man who sees in it a method of wiping out the disease at one stroke. He is strengthened in this idea by the expressions which are used such as tuberculosis-free area,

tuberculosis eradication. It has an appeal to the sanitarian for it seems to promise a solution of the public health phase of the problem. It appeals to the legislators for it seems to offer an ultimate escape from a heavy financial load.

There are a number of phases of the work that merit consideration. First, what is the hope of ultimate success? It seems to me that it is small, except as the work can be continued for a considerable term of years. I believe it can be done, but it will require much more effort, time and money than most are willing to admit. This means, does it not, that the hopes of multitudes of people are being based on false premises, on the infallibility of the tuberculin test and when these show themselves to be false, those interested swing to the other extreme and say there is no use and refuse to carry on. There are signs of this already.

The area test has so far been limited to sections in which the disease is not widespread, those in which 0.5 to 5 per cent of the animals are tuberculous. There are three counties in this country that have been tested and retested. One in Michigan with 5 per cent of the animals reacting on the original test and 1.8 per cent on a retest one year later. Two in Wisconsin with 0.8 and 1 per cent reactors on the original test and approximately the same on a retest three years later. It will probably demand a decade of persistent work to eradicate tuberculosis from these counties which at present contain approximately 100,000 dairy cattle. Can the interest be maintained at its present level for this period? It seems doubtful, for it seems possible that at each reëxamination of the cattle an appreciable part may react, even though no tuberculosis can be demonstrated on postmortem examination.

For many years it was asserted by those high in authority that the tuberculin test was well nigh infallible. It was claimed that less than 1 per cent of reacting animals showed no lesions on slaughter. Last year approximately 8 per cent of animals reacting to tests made under the most favorable conditions by trained men failed to show lesions of tuberculosis.

It has been shown that as the percentage of reacting animals decreases, the percentage of no-lesion animals increases. This is exactly what one would expect if some related organism were present which sensitized the animal to tuberculin. The acid fast bacilli found in the skin lesions are apparently of this nature.

We have had under observation a herd from which reacting animals have been removed year after year and in no case have lesions been found. In only one instance were acid fast bacilli found, in an abscess of the lungs due to mechanical causes. Is this herd one in which some organism closely related to the tubercle bacillus has established itself?

The magnitude of the errors on the other side; namely, the non-recognition of the tuberculous animal through the tuberculin test is almost an unknown factor. It is surprising to find how little has been done to determine their magnitude. The non-recognition of a tuberculous animal is likely to prove of far greater importance than the removal of a healthy animal from a herd on the suspicion that it is diseased.

As was stated previously, the percentage of reacting animals on a retest of areas in Wisconsin containing over 80,000 cattle showed as high a percentage of reacting animals as did the original test. These results can be explained only through the leaving of tuberculous cattle in the herds, which we know is a possibility, or through the interference of some other than the tubercle organism with the tuberculin test, something of which we know nothing. From either point of view our method is so lacking that its faults may prove a serious handicap to the tuberculosis limitation movement.

There is still another phase of this same problem which again may retard its solution, and that is the financial side. There must be available large sums of money to carry on this work, since the testing must be continuous.

The money for this work is derived from the public funds, both for operation and for the indemnification of owners whose animals are found diseased. I suppose the sum available for this work will amount during the current year to nearly $10,000,000. This money is being spent primarily for the pro-

motion of public health, because the bacteriologist has determined that a portion of the disease in man is due to organisms from bovine sources.

It seems to me that it is a fair question to ask whether these monies are being wisely spent from the standpoint of the health of the people. If the returns from the standpoint of public health do not justify the expenditure of public funds, then certainly it should not be continued, for in no other line are the public funds used to free an industry from a trouble that is an economic one. I feel quite certain that an equal amount of money expended in other ways would bring a much greater result in prevention of disease and in the saving of human life. The data concerning the percentage of human tuberculosis due to bovine origin have been collected, wholly, I think I may say, in parts of the world in which bovine tuberculosis is much more prevalent than in most areas of our country. It would thus seem that to obtain any adequate picture of the sanitary importance of the disease in this country the usual figures must be divided by 2 or 3 and possibly by 5. It may be that the heavy expenditure of public funds may not be justifiable.

The law makers have accepted at face value the statements of bacteriologists. A recognition of the fact that these statements may not adequately picture the situation may result in a reaction against the present methods and leave conditions far worse than before, because to start a movement a second time is more difficult than at first.

May it not be wise to recognize that bovine tuberculosis is primarily an economic problem that whatever of public health aspects it possesses are being cared for more and more completely each year through the pasteurization of milk for direct consumption and of cream for butter making.

The people living in the cities have little to fear from bovine infection, and the farmer should be willing to protect the health of his own family, especially when this protection is likely to prove of financial advantage to him. I say likely for I am afraid no one can demonstrate to the ordinary milk producer what importance bovine tuberculosis plays in his financial

success. I mean by this can we say that on the average the tuberculous herd will be 1 per cent or 10 per cent or 25 per cent less efficient than the tuberculosis free herd. This information is what the modern business-like farmer demands if he is to act.

It was originally thought that the healthy animal was the important thing. Somewhat later it was realized that the healthy herd was the unit to consider in tuberculosis, and only very recently is the importance of the tuberculosis-free community beginning to be felt. Many are coming to see that the health of his neighbor's herd is almost as important to him as is that of his own. It will be recognized that tuberculosis is a community problem of the cattle industry and of importance to those outside this industry only in an indirect way. If this is true, should not the funds needed to carry on the work be derived from those in the industry and not from the public at large. The funds must, however, be spent under public control, for in no other manner can the unity of method essential to success be attained.

The future must bring, it seems to me, a mutual protective association of the cattle owners which shall tax its members in proportion to their interest for the purpose of raising a fund which shall be used to do such testing as is necessary, and to indemnify the members for losses sustained. A mutual tuberculosis insurance company under state control, if you will, and with this will come the release of public funds for more renumerative purposes.

I am fully aware that there are many things to be considered. For example, if through the expenditure of a large amount of money during a few years, we could protect the public against the danger of bovine tuberculosis for a long time in the future, the money would be well expended.

Any change in our methods of approaching the problem of bovine tuberculosis will have to be initiated by the bacteriologist, rather than by the public health official, the live stock sanitarian, or the practicing physician or veterinarian, and hence it seems to me worth while to present it to a group of bacteriologists.

There is still another method used widely in bacteriological work which may have limitations in directions not widely recognized at present. I refer to the agglutination reaction, which is said to be a general biological phenomenon. For many years it has been widely used both for diagnosis and for the identification of bacteria. In both of these uses it has been considered to have few limitations, and these have been thought of as being due to non-specificity. As for example with the typhoid-paratyphoid group, group agglutinins are developed which in low concentrations of the immune serum may lead to confusion. With higher dilutions the point is usually reached when the group agglutinins are so diluted as not to exert an action, while the specific agglutinin is still present in amounts which will enable it to be easily recognized. I am not aware that the observation has been made that there may be limitations in the opposite direction and that the reaction may be ultra specific. I can make this clear by presenting briefly some results obtained by Mr. Stevens in the laboratory with which I am connected.

The nodule-forming bacteria of the leguminous plants are divided into a number of groups, depending upon the kinds of legumes they are able to infect, meaning by this the power to invade, to cause the development of nodules, and to enable the plant to draw on the free nitrogen of the air for its supply. The bacteria isolated from the nodules of any plant of the group are able to infect any other member of the group. Earlier work had shown that the bacteria related to these groups were mutually exclusive in relation to the agglutination reaction, a result to be expected from the knowledge gained in the study of other bacteria. We were surprised to find that each of these groups as determined by the plant could be divided into a number of sub-groups by the agglutination test. In other words the injection of an animal with a culture coming from alfalfa will stimulate the production of agglutinins which will produce complete agglutination in dilutions as high as 1 to 30,000 with many other cultures derived from alfalfa. The serum of this immunized animal may not agglutinate still other cultures coming from alfalfa in even such low dilutions as 1 to 20. The

question at once arises, Have we not in hand different bacteria? It has seemed to us that the final answer to this question is found in the relation of the two cultures to the alfalfa plant. If each of these cultures is brought in contact with the young plant under conditions that preclude any errors and nodules are produced by both cultures, and both enable the plant they have invaded to draw upon the atmospheric nitrogen, it would seem we are justified in saying that the cultures are identical in nature in spite of the fact that the agglutination tests show them to be different. It may be that we are placing too much reliance on the relation of the cultures to the higher plants, but it has seemed to us that this is a firmer basis than any cultural, morphological or biochemical characteristic for the nature of the organism.

If our ideas are correct as to the identifying value of the relation between green plant and bacteria, the results raise a host of questions in regard to other groups of bacteria. Are subgroups to be met among the typhoid bacteria? If so, may it not be that our diagnostic measures enable us to recognize but a part of the cases of typhoid, since if a case is due to a member of another sub-group than the one used in making the test no positive result would be obtained, or again a suspected culture would not be recognized as typhoid if it failed to agglutinate with the serum of an animal treated with a member of another sub-group. Please understand that I am not saying this is the case, but only indicating that it may be a possibility, judging from the results obtained with a group of bacteria to which the immunologists have devoted no attention; and yet it is a group which we believe has no equal for the purpose, for no other group can be identified with the same degree of certainty.

I have often felt that the medical bacteriologists and immunologists could work to advantage with organisms which at first glance might seem foreign to their interests.

There are certain conditions that make difficult progress in regard to methods. The research man who is using a method as an aid in his work has not the same opportunity to determine its limitations as has the man who is using the method in a

practical way. The clinician, the physician, the veterinarian, the control laboratory worker have more opportunities to gauge the value of a method if they will live up to their opportunities. I am afraid that most of those engaged in the practical applications have full faith in the methods handed to them and rarely question the value of the information gained through their use. I think this is a very natural attitude for other wise they could not be happy in their work. They would be constantly assailed by the thought that they were not giving correct information to their clients. The happiest servant, whether he be physician, lawyer, or clergyman, is he who is certain the advice he is giving is the most valuable that could possibly be bestowed. Research and practice are somewhat incompatible.

If all interested in bacteriology are wide awake to the limitations of our tools, it will not be long before they are much improved and we shall feel more certain of the structures built therewith.

STUDIES OF THE STREPTOCOCCI

VII. A MEDIUM FOR STOCK CULTURES OF STREPTOCOCCI AND OTHER BACTERIA

S. HENRY AYERS AND WM. T. JOHNSON, JR.

From the Research Laboratories of the Dairy Division, United States Department of Agriculture

Received for publication July 5, 1923

For several years we have been carrying a large number of stock cultures of streptococci, among which were many species, both pathogenic and nonpathogenic. When transplanted on to infusion agar some of the cultures grew quite rapidly, others grew with difficulty, and almost all died rapidly. This necessitated frequent transfers, requiring considerable time to keep the cultures in an actively growing condition.

Several years ago Dr. G. W. Supplee, then at the New York State College of Agriculture, very kindly sent us a formula for a medium for streptococci, which contained the following ingredients:

	per cent
Meat infusion	
Gelatin	2
Agar	1
Pepton	2
Nutrose	0.5 per cent (or 1 per cent glucose)
Reaction adjusted to neutral with phenolphthalein	

This medium gave good results as far as the growth of the streptococci was concerned, and was very helpful in carrying stock cultures. The life of the cultures, however, was not widely different from what would be found in plain infusion agar. Many experiments have been conducted with this medium with a view to improving it both from the standpoint of growth and the length of life of organisms growing upon it.

This experimental work has resulted in producing a medium, differing considerably from the original, which has given excellent results, not only with streptococci but with other organisms as well.

THE MEDIUM

The ingredients of the medium are as follows:

	per cent
Meat infusion	
Pepton (Parke, Davis)	1.0
Gelatin (Difco)	1.0
Casein (pure, prepared according to Hammarsten)	0.5
Glucose	0.05
$Na_2HPO_4+2H_2O$ (Sorrenson's phosphate)	0.4
Sodium citrate	0.3
Agar	0.75

The preparation of the medium is simple if carried out according to the following method:

PREPARATION OF 1 LITER

(A)

Meat infusion broth	500 cc.
Pepton (Parke, Davis)	10 grams
$Na_2HPO_4+2H_2O$ (Sorrenson's phosphate)	2 grams

Heat until dissolved and then adjust reaction to pH 7.8.

(B)

Distilled water	150 cc.
Casein (pure, prepared according to Hammarsten)	5 grams
$Na_2HPO_4+2H_2O$ (Sorrenson's phosphate)	2 grams

Heat until dissolved, add to A, and to the resulting mixture of A and B add 10 grams of gelatin (Difco).

(C)

Heat the mixture A plus B, after adding the gelatin, in the autoclave at 15 pounds, for 10 minutes.

Add 0.5 grams glucose.

The reaction at this point should be pH 7.6.

Filter through paper.

(D)

Prepare 250 cc. of 3 per cent agar. Filter through cotton and dissolve 3 grams of sodium citrate in the melted agar.

(E)

Mixed agar and filtered solution from C and make up to 1000 cc. by adding distilled water.

(F)

Tube and sterilize for twenty minutes at 15 pounds in the autoclave. After sterilizing allow tubes to cool slowly. If a slight precipitate has formed it will settle or be absorbed in a short time; in either case the medium is not affected. The final pH is 7.5.

GROWTH AND LIFE OF CULTURES IN THIS MEDIUM

All species of streptococci in our collection, including the pathogenic types, show a remarkably heavy growth in this medium. For carrying stock cultures, the medium is not slanted; stabs are made by inoculating with a loop which is passed into the center of the tube of solidified medium nearly to the bottom. Stab cultures are made in preference to slants because of the greater evaporation from slants, but in dealing with strictly aerobic cultures slants may be used.

It has been found that streptococci, including the usual delicately growing pathogenic species, remain alive in this medium when held at room temperature averaging 75°F. for a period of at least four months. The experiment had to be stopped after four months because the medium had practically dried up, although viable cultures could still be obtained from this dry mass. In this experiment the tubes were exposed with ordinary cotton plugs; if tightly stoppered or refrigerated, the life of the cultures would undoubtedly have been greatly increased.

We have received very encouraging reports from other investigators who have used this medium. They like it for the reason that very delicate cultures will survive for two months on it, while on ordinary media they live from three to fourteen days at the most. Mr. W. R. Albus, of these laboratories, has grown the entire museum collection of the Society of American Bacteriologists on this medium with no trouble. Especially is this notable in that he did not have to transfer *Diplococcus pneumoniae* more often than every four weeks, and found that *Hemophilus pertussis* grew better on this casein medium than

on the regular medium prescribed for it. Dr. L. T. Giltner, of the Pathological Division, of the Bureau of Animal Industry, of this Department, reports that he grew the human tubercle bacillus, also *Pasteurella bovis*, *Erysipelothrix porci*, and many other poorly growing organisms on the medium. The greatest benefit derived from its use was the less frequent transferring required to keep his stock cultures active.

The luxuriant growth and long life of the cultures grown on this medium is probably due to the consistency of the medium, the presence of casein, the slight amount of sugar which serves as an easily available source of carbon, and the favorable buffering of the medium.

STUDIES OF THE STREPTOCOCCI

VIII. A NOTE ON HYDROGEN-SULPHID PRODUCTION BY STREPTOCOCCI

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The ability of certain species of bacteria to produce hydrogen sulphid from culture media has been extensively studied, but in such work the streptococci seem to have been neglected. Gordon (1910), however, states that *Streptococcus fecalis* produces hydrogen sulphide and Dible (1921), also, has noted this fact. We have, on numerous occasions, observed evidence of hydrogen-sulphid production by several different species of streptococci.

The interesting work of Tilley (1923), on the production of hydrogen sulphid from sulphur compounds led us to try similar experiments with several different species of streptococci.

In our experiments the following medium and method for determining hydrogen-sulphid production were used. The medium consisted of infusion broth with 1 per cent of Parke-Davis pepton, the reaction adjusted to pH 7.5. To this basic medium sulphur compounds were added in a proportion of 0.25 per cent as used by Tilley (1923). For the determination of hydrogen sulphid the method of Jordan and Victorson (1917) was used as follows: A 10 per cent solution of lead acetate was prepared and sterilized in the autoclave. This solution was added in the proportion of 0.1 per cent, or from 2 to 3 drops to each tube of medium which had been melted and allowed to cool to about 43°C. The tube was thoroughly shaken and the agar allowed to solidify. The tubes were then inoculated by pushing the needle down the side between the agar and the glass.

The ability of five different species of streptococci and of *Bacterium coli* to produce hydrogen sulphid was tested, with results shown in table 1. The colon culture was used as a control.

Although the reactions recorded were observed after four-weeks incubation at 30°C., the presence of hydrogen sulphid, as evidenced by blackening of the medium, was shown within twenty-four to forty-eight hours. No hydrogen sulphid was produced by the streptococci in the basic medium without added sulphur. However, all the cultures except the *Streptococcus kefir* formed hydrogen sulphid from the thiosulphate.

TABLE 1

Hydrogen-sulphid production

NAME OF CULTURE	NO ADDED SULPHUR	SODIUM THIOSULPHATE	SODIUM THIOSULPHATE WITH 0.5 PER CENT DEXTROSE	SODIUM SULPHITE	AMMONIUM SULPHATE
Streptococcus pyogenes	−	+++	+	−	+
Streptococcus mastiditis	−	++	+	−	−
Streptococcus lactis	−	++	+++	−	−
Streptococcus kefir	−	−	+	−	−
Streptococcus bovis	−	+	++	−	−
Bacterium coli	++	++++	++++	++	++

Reactions recorded were taken after four weeks incubation at 30°C.

− = no hydrogen sulphid; + = trace; ++ = small amount; +++ moderate amount; ++++ = large amount.

It will be noted that different amounts of hydrogen sulphid were produced and that *Streptococcus pyogenes* showed the largest amount and *Streptococcus bovis* the least. The addition of 0.5 per cent glucose to the medium seemed to influence hydrogen-sulphid production, as can be observed.

It is apparent, therefore, that streptococci can form hydrogen sulphid from partly oxidized sulphur in the form of the thiosulphate.

Negative results were obtained with sodium sulphite; that is to say, no hydrogen sulphid was formed. In this case growth was inhibited to a large degree, and the inability to produce hydrogen sulphid may be due to this fact.

Streptococcus pyogenes was the only streptococcus which gave any evidence of producing hydrogen sulphid from oxidized sulphur in the form of the sulphate, and it showed only a trace.

In another series of experiments plates were made with the lead-acetate medium plus the thiosulphate, and were inoculated by drawing a needle in a line through the medium. These plates incubated at 30°C. showed interesting results. *Streptococcus pyogenes* showed a blackening along the line of inoculation in twenty-four hours, and *Streptococcus mastiditis* a faint blackening in forty-eight hours. None of the other streptococci showed any evidence of hydrogen-sulphid production, although growth was abundant. These results were somewhat different from those obtained in tubes, and may be due to the aerobic conditions in the case of growth on plates. In the tubes it was observed that blackening began to appear along the line of growth toward the bottom of the tube.

From previous experiments in which unoxidized sulphur in the form of cystin was used, it was found that many streptococci form hydrogen sulphid. Results were also obtained by Dr. C. S. Mudge, formerly of these laboratories, which indicated that hydrogen sulphid could be produced from colloidal sulphur.

CONCLUSIONS

It has been shown that certain species of streptococci are able to produce hydrogen sulphid from sulphur compounds.

No attempt has been made to apply this test for classification purposes, but it seems likely that the production of hydrogen sulphid under controlled conditions, and perhaps a quantitative determination of the amounts formed, may serve a useful purpose.

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THE MOTILITY OF BACTERIA AS EFFECTED BY HYDROGEN ION CONCENTRATIONS

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It has recently been shown in this laboratory that the hydrogen ion concentration of culture media has a pronounced effect upon the structure of bacteria (1). It appeared probable therefore, that the flagella, being the most exposed part of the protoplasm, would be first affected by changes in acidity. As the condition of the flagella is evidently indicated by the motility of the organism it seemed that a measure of the motility of organisms exposed to various hydrogen ion concentrations would provide a delicate measure of the influence of this condition upon the protoplasm. A study has been made of the motility of two species, *Bact. typhosum* and *Ps. pyocyanea* when grown or suspended in solutions of different acidities.

Several methods which have frequently been used in the study of motility were attempted but were abandoned in favor of the direct observation of the motility of the organisms in the conventional hanging drop. The hanging drops, made in closed cells, were placed in a warm stage and kept at a temperature of $37° \pm 2°$. Observations were made upon the central portion of the drop only, by moving to the center of the drop on the coverglass and focusing to a point midway between the underside of the glass and the bottom of the drop, as in that region it was found there was least surface tension influence upon the free movements of the organisms. To determine the percentage of motility the number of motile and nonmotile organisms present in one ruled

[1] Holder of a bursary from the Canadian Council of Industrial and Scientific Research.

square of the ocular during the course of one minute was noted and an average made of twenty different areas in the drop taken at random.

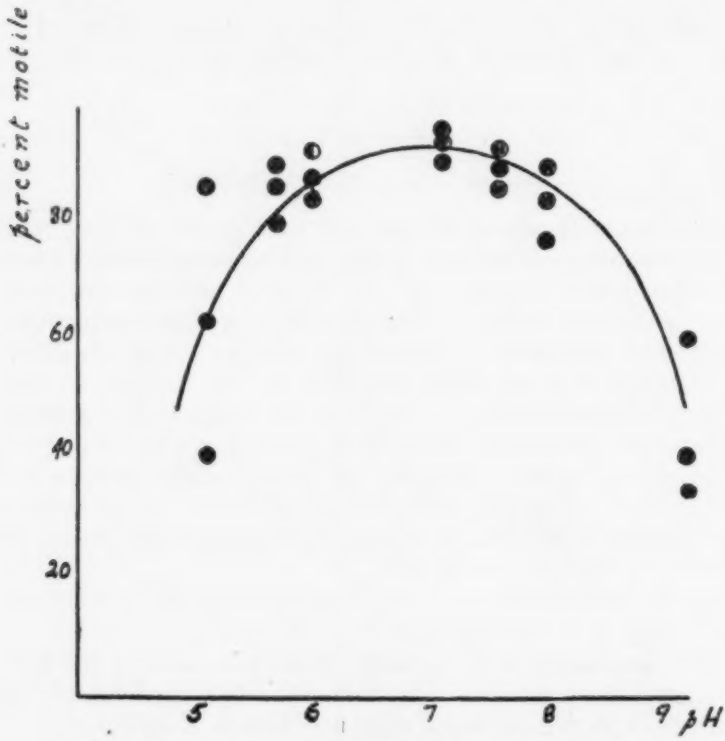

FIG. 1. CURVES SHOWING THE MOTILITY OF BACT. TYPHOSUM

The ordinates represent the percentage of organisms motile, the abscissa the pH of salt solutions in which the organisms were suspended for examination.

The organisms were grown and examined in nutrient broth of beef extract and pepton buffered with phosphates to pH 5.0 to 8.0. Or they were grown on the surface of neutral nutrient agar and suspended in solutions of sodium chloride buffered with acetates, phosphates or borates to pH 3.5 to 10, a much wider range than could be used in the former case. The pH of the

solution was found to have a similar effect upon the motility of the organisms whether acting in a nutrient solution in which the organisms were grown or in the solution in which they were suspended.

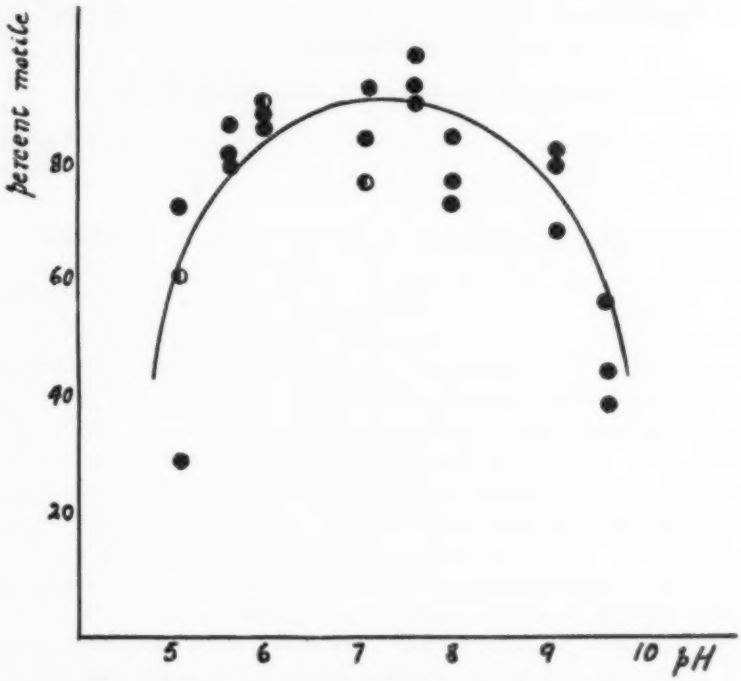

FIG. 2. CURVES SHOWING THE MOTILITY OF PS. PYOCYANEA

The ordinates represent the percentage of organisms motile, the abscissa the pH value of salt solutions in which the organisms were suspended for examination.

The results obtained are indicated in figures 1 and 2. *Bact. typhosum* was found to possess a maximum motility between pH 6.0 and 8.0; progressively less motility between pH 6.0 and about 4.5 and between 8.0 and about 9.5. No motility was observed beyond these limits. *Ps. pyocyanea* gives essentially the same motility curve except that the organisms withstand a slightly higher alkalinity, maintaining some motility up to pH 10.0.

Comparison of these motility curves with the familiar curves of growth in relation to hydrogen ion concentration indicates that the motility of these two species is influenced by the reaction of the media in essentially the same manner as growth is influenced.

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THE QUANTITATIVE ACTION OF ENZYMES OF SEVEN SPECIFIC ORGANISMS UPON THE PROTEINS OF MILK AND UPON GELATIN

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The object of this work was to determine quantitatively the action of proteolytic enzymes of common milk organisms upon the proteins of milk and upon gelatin.

Little is known in regard to the microbial proteolytic enzymic action of the milk organisms upon the proteins of milk and upon gelatin. Our interest in this work was brought about in studying the effect exerted by specific organisms, when inoculated into sterile butter, upon the keeping quality of the butter. One of the measures used in determining the keeping quality was the amount of protein decomposition. It was found that after the death of some of the organisms proteolytic action continued. This led us to believe that the enzymes secreted by the organisms continued the reaction. In studying the enzymic action, milk was used, instead of butter, on account of the difficulty in working with butter and because the small amount of protein contained in butter increases the experimental error.

Work previously done by the authors, and that of Combs and Eckles (1907), has shown that a temperature of 64.5°C. inactivates only a very small percentage, less than 8 per cent of the enzyme peroxidase, when the test medium is held at that temperature for twenty minutes and then cooled. This suggests that enzymes may be carried over from the cream before pasteurization to the resulting butter. Much cream for buttermaking is pasteurized by the flash method; an experiment was therefore conducted to determine the effect of flash pasteurizing upon the

enzyme peroxidase. The apparatus was so arranged that the temperature could be obtained in about three seconds.[1]

TEMPERATURE	PEROXIDASE DESTROYED
°C.	*per cent*
85	92.5
80	42.5
76	40.0
64	Very slight

This table shows that flash pasteurizing at 85°C. inactivates a large percentage of the enzyme peroxidase.

It might reasonably be expected that the effect of heat on the enzyme peroxidase would in a general way be paralleled in the case of many proteolytic enzymes. The optimum temperature for enzyme action is about 50°C. No doubt a slight inactivation begins at this temperature, but it is well known that an increase of temperature increases chemical reaction so that at the optimum temperature the rate of chemical changes brought about has reached its maximum. At about 50°C. the enzyme activity diminishes gradually until the temperature rises to 70°C.; then the activity of the enzymes diminishes quite rapidly and at 85° to 90° is generally completely destroyed. In view of the above results it was deemed advantageous to continue the work on enzymes of specific organisms as they affect the proteins of milk and gelatin.

Organisms used were obtained from reliable sources and rechecked in order fully to determine their correct nomenclature. Milk cultures were made every twenty-four hours for seven periods; this was done to activate the organism so that it would be similar to organisms as found under normal conditions. After seven transfers, an inoculation of 0.1 cc. was made into 500 cc. of sterile beef broth with a reaction of pH 7.0, incubated at 33°C. for forty-eight hours and filtered through a Mandler filter. The filtrate was then incubated for eighteen hours at 33°C. in order to determine its sterility. If no growth was apparent, the filtrate

[1] Spitzer, George and Taylor, W. C. (Manuscript in preparation.)

remaining clear, 10 cc. portions were added to 250 cc. of sterile milk and sterile 2 per cent gelatin solutions, reactions of media pH 6.9. The control or blank was inoculated with 10 cc. of sterile beef broth. Flasks were then incubated at 33°C. for definite intervals and prepared for chemical analysis. Previous to chemical analysis each flask was examined by removing 5 cc. and inoculating into a nutrient broth tube. Turbidity after twenty-four hours meant contamination and the flask was discarded. Only flasks showing no turbidity after twenty-four hours were submitted to chemical analysis. The contents of the check were analyzed at each interval, and no apparent change took place in the preceding groups.

The chemical analysis consisted of determining the per cent of amino acids and peptons. This was done by determining the amount of nitrogen in the filtrates of zinc sulphate and phosphotungstic acid and calculating the per cent pepton by the difference in the per cent of nitrogen. This work was done in duplicate, and in the following manner.

Precipitation with zinc sulphate. To the solution to be analyzed sufficient dilute sulphuric acid was added so as to make it distinctly acid to methyl red. The solution was slightly warmed and chemically pure zinc sulphate added to saturation (a portion of the zinc sulphate crystalized when the solution was cooled to room temperature). After standing for twenty-four hours at room temperature, the solution was filtered and the nitrogen determined in the filtrate, this representing nitrogen not precipitated by zinc sulphate.

Precipitation with phosphotungstic acid. To the neutralized solution to be analyzed, sufficient sulphuric acid was added to bring the acidity to 5 per cent. Sufficient 5 per cent phosphotungstic acid solution was added and the mixture allowed to stand for twenty-four hours at room temperature; the solution was then filtered and the nitrogen determined in the filtrate,—representing the nitrogen not precipitated by phosphotungstic acid, sometimes considered as amino acids.

Table 8 is a summary of tables 1 to 7, showing the proteolytic action on milk and gelatin of the enzymes produced by the

TABLE 1

The action of the proteolytic enzymes of B. mesentericus on the proteins of milk and on gelatin

	MILK				GELATIN			
	N not PPT by P.T.A.	N not PPT by $ZnSO_4$	Pepton	Total N	N not PPT by P.T.A.	N not PPT by $ZnSO_4$	Pepton	Total N
Check	0.0202	0.0378	0.0176	0.520	0.0160	0.0232	0.0072	0.340
0 hours	0.0224	0.0400	0.0176		0.0200	0.0280	0.0080	
24 hours	0.0236	0.0425	0.0189		0.0220	0.0308	0.0088	
72 hours	0.0254	0.0466	0.0212		0.0238	0.0378	0.0140	
120 hours	0.0308	0.0492	0.0184		0.0294	0.0492	0.0198	
168 hours	0.0322	0.0514	0.0192		0.0322	0.0574	0.0252	

N not PPT by P.T.A. = nitrogen not precipitated by phospho-tungstic acid.
N not PPT by $ZnSO_4$ = nitrogen not precipitated by zinc sulphate.

TABLE 2

The action of the proteolytic enzymes of B. mycoides on the proteins of milk and on gelatin

	MILK				GELATIN			
	N not PPT by P.T.A.	N not PPT by $ZnSO_4$	Pepton	Total N	N not PPT by P.T.A.	N not PPT by $ZnSO_4$	Pepton	Total N
Check	0.0200	0.0234	0.0034	0.467	0.0112	0.0182	0.0070	0.325
24 hours	0.0280	0.0504	0.0224		0.0112	0.0840	0.0728	
72 hours	0.0364	0.0700	0.0336		0.0224	0.0882	0.0658	
120 hours	0.0392	0.0896	0.0504		0.0308	0.1106	0.0788	
192 hours	0.0378	0.0952	0.0574		0.0340	0.1652	0.1312	

TABLE 3

The action of the proteolytic enzymes of B. ichthyosmius on the proteins of milk and on gelatin

	MILK				GELATIN			
	N not PPT by P.T.A.	N not PPT by $ZnSO_4$	Pepton	Total N	N not PPT by P.T.A.	N not PPT by $ZnSO_4$	Pepton	Total N
Check	0.0203	0.0322	0.0119	0.445	0.007	0.0154	0.0084	0.420
48 hours	0.0294	0.0952	0.0658		0.0154	0.126	0.1102	
96 hours	0.0476	0.126	0.0784		0.0182	0.140	0.1318	
142 hours	0.0513	0.1358	0.0845		0.0357	0.1932	0.1575	
190 hours	0.0612	0.1470	0.0858		Sample contaminated			

TABLE 4

The action of the proteolytic enzymes of B. aerogenes (ropey) on the proteins of milk

	MILK			
	N not PPT by P.T.A.	N not PPT by $ZnSO_4$	Pepton	Total N
Check	0.0200	0.0234	0.0034	0.467
24 hours	0.0224	0.0252	0.0028	
72 hours	0.0334	0.0474	0.0140	
120 hours	0.0340	0.0492	0.0152	
192 hours	0.0378	0.0566	0.0188	

TABLE 5

The action of the proteolytic enzymes of S. cremoris viscosus on the proteins of milk and on gelatin

	MILK				GELATIN			
	N not PPT by P.T.A.	N not PPT by $ZnSO_4$	Pepton	Total N	N not PPT by P.T.A.	N not PPT by $ZnSO_4$	Pepton	Total N
Check	0.0203	0.0322	0.0119	0.445	0.007	0.0158	0.0084	0.420
48 hours	0.0252	0.0490	0.0338		0.0105	0.0910	0.0805	
96 hours	0.0308	0.0756	0.0445		0.0126	0.1344	0.1218	
142 hours	0.0336	0.0910	0.0574		0.0189	0.1666	0.1477	
190 hours					0.0224	0.1778	0.1554	

TABLE 6

The action of the proteolytic enzymes of E. prodigiosus on the proteins of milk and on gelatin

	MILK				GELATIN			
	N not PPT by P.T.A.	N not PPT by $ZnSO_4$	Pepton	Total N	N not PPT by P.T.A.	N not PPT by $ZnSO_4$	Pepton	Total N
Check	0.0203	0.0322	0.0119	0.445	0.007	0.0158	0.0084	0.420
48 hours	0.0206	0.0658	0.0452		0.0084	0.0728	0.0644	
96 hours	0.0220	0.0660	0.0446		0.0147	0.148	0.1333	
144 hours	0.0247	0.0688	0.0441		0.0189	0.1694	0.1505	
216 hours	0.0308	0.0826	0.0518		0.0252	0.1918	0.1666	

TABLE 7

The action of the proteolytic enzymes of B. albolactis on the proteins of milk and on gelatin

	MILK				GELATIN			
	N not PPT by P.T.A.	N not PPT by Zn SO_4	Pepton	Total N	N not PPT by P.T.A.	N not PPT by Zn SO_4	Pepton	Total N
Check	0.0252	0.0378	0.0126	0.518	0.0070	0.0252	0.0182	0.331
48 hours	0.0294	0.0560	0.0270		0.0168	0.0700	0.0532	
72 hours	0.0376	0.0896	0.0520		0.0168	0.154	0.1372	
144 hours	Sample contaminated				0.0224	0.2716	0.2492	
192 hours	0.0426	0.1190	0.0764		0.0322	0.273	0.2408	
240 hours	0.0420	0.1260	0.0840		0.0392	0.280	0.2408	

TABLE 8

SOURCE OF ENZYME	MILK		GELATIN		
	Amino acid	Pepton	Amino acid	Pepton	Hours digested
B. mesentericus	1.88	0.40	3.59	5.06	168
B. mycoides	3.82	11.64	7.14	38.21	192
B. ichthyosmius	9.19	16.58	6.84	35.50	190
B. aerogenes	3.82	3.30			192
S. cremoris viscosus	2.99	10.22	3.67	35.00	190
E. prodigiosus	2.36	8.97	4.34	37.66	216
B. albolactis	3.26	13.78	9.73	66.96	240

respective specific organisms. The figures express the increases in nitrogen compounds not precipitated by phosphotungstic acid, and the difference of the nitrogen compounds not precipitated by a saturated solution of zinc sulphate and phosphotungstic acid, designated as peptons and based on total nitrogen.

In discussing the enzymic action of the organisms mentioned, there is a notable uniformity in the quantity of amino acids produced during the respective periods of digestion. The exceptions are the action of the enzymes produced by *B. mesentericus* and *B. ichthyosmius*. The enzymes of *B. mesentericus* produced the least proteolytic action, while the enzymes derived from *B. ichthyosmius* produced the greatest quantity of amino acids of any of the enzymes studied, likewise a large quantity of pepton in both milk and gelatin.

The enzymes derived from *B. aerogenes* produced the least amount of pepton in milk, except for the enzymes of *B. mesentericus*. The most significant part of the results of this study is the difference of the enzymic action on milk proteins and on gelatin. It can be seen that the amount of pepton produced by the enzymes from different organisms is approximately four times greater in gelatin than in the case of pepton produced in milk.

The remarkable fact is that the rate of peptonization of gelatin per hour is practically constant, being approximately 0.2 per cent, except for *B. mesentericus*. A slight exception is to be made for *B. albolactis* the figure in this case being a little more than 0.25 per cent. The difference of the proteolytic action on milk proteins and gelatin can in a great measure be accounted for with some degree of certainty. Spitzer and Epple (1920), studied the character and proteolytic properties of the enzymes produced by *B. panis* (Migula) on purified casein. It was found that these enzymes produced the optimum amount of amino acids and pepton in neutral solutions, less action being manifest in a 0.2 per cent acid solution. The following figures were obtained.

REACTION	NITROGEN NOT PRECIPITATED BY $ZnSO_4$	NITROGEN AS PEPTON
Action of enzymes of *B. panis* upon purified casein		
Neutral	60.75	35.40
0.2 per cent acid	16.55	11.45
Action of enzymes of *B. panis* on egg albumin		
Neutral	44.30	28.50
0.2 per cent acid	19.00	12.15

The digestive period was the same for casein and egg albumin.

It is reasonable, therefore, to expect a change in the milk protein solution; as milk contains lactose some acidity may be produced retarding the action of certain specific enzymes, especially the peptonizing enzymes. According to Marshall

(1921), and Euler and Pope (1912), fermentations are produced by enzymes derived from microörganisms and some of these enzymes may produce acidity.

Acknowledgment is due to W. F. Epple.

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THE FERMENTATION OF GALACTOSE BY SACCHAROMYCES CEREVISIAE

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From the researches of Dienert (1900), and Kluyver (1914) it had appeared that many yeasts which initially failed to ferment galactose, did so in course of time, or in other words, that adaptation occurs. From Kluyver's data it is apparent, that the newly formed cells have acquired the property of fermenting galactose. This acquisition of the power to ferment galactose being a rather important fact from a biological view-point, it was further studied, in connection with the findings of previous investigators.

THE CONNECTION BETWEEN THE ADAPTATION AND THE GROWTH OF YEAST

As was the case with Kluyver, we repeatedly ascertained, that at a temperature of 38°C., at which no reproduction takes place, carbonic acid could never be obtained from galactose. The following experiments were made, in order to trace the correlation between an increase in the quantity of yeast and the galactose-fermentation-rate. For all our experiments we used the compressed yeast of the Dutch Yeast- and Spirit-works at Delft.

Experiments were made to determine the connection between the galactose-fermentation-rate at 38°C., and the time for which the yeast had an opportunity of multiplying at 30°C., and likewise the connection between the galactose-fermentation-rate at 38°C. and the number of yeast-cells. The fermentation-rate was determined by shaking in a water-bath at 38°C. and measuring every 6 minutes the developed quantities of carbonic acid,

received in burettes, over a saturated salt-solution. Owing to the temperature, the quantity of yeast remains unaltered during this experiment. The growth of the yeast was brought about by transferring it to a water-bath of 30°C. in which an equable shaking occurred. The number of yeast-cells was determined under the microscope, viz., by the average number of cells per partition of the Fuchs-Rosenthal counting-chamber (average of 48 counts per fermentation-vessel). The subjoined table shows the experimental results (the figures represent the means of fermentation-vessels).

PERIOD OF SHAKING AT 30°C.	SUBSEQUENT RATE AT 38°C.	AVERAGE NUMBER OF CELLS PER PARTITION
0 minutes.....	0	8.4
50 minutes.....	0	0
2 hours 10 minutes.....	0.6	0
3 hours 20 minutes.....	2.3	13.3
4 hours 10 minutes.....	3.2	14.8
5 hours 40 minutes.....	5.5	18.6
6 hours 35 minutes.....	6.0	19.2
7 hours 30 minutes.....	6.3	20.0

See graphical representation 1 *a* and 1 *b*. From the proportion between the increase in the number of yeast-cells, grown in galactose-solution, and the increase in the fermentation-rate, as shown by the straight line in figure 1 *b*, it follows that the newly formed cells have acquired the property of fermenting galactose; the old cells did not obtain this property, for if this were the case, the line in 1 *b* should show an ascending curve growing constantly steeper, instead of a straight line (at the end of the experiment the percentage of old cells still amounts to 42). The figure in 1 *a* reminds us of an individual variation-curve. Analogous experiments gave corresponding results.

THE ENZYMATIC CHARACTER OF THE NEW PROPERTY

When we examine carefully the same yeast, partly cultivated in a glucose-solution, partly in a galactose-solution, with respect to galactose-fermentation at 38°, the latter will at once liberate carbonic acid from this sugar, the former never. The question

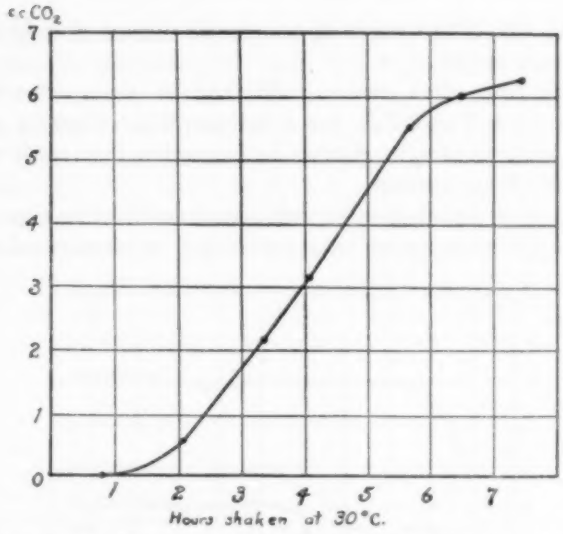

FIG. 1A. GALACTOSE FERMENTATION RATE, CUBIC CENTIMETERS CO_2 PER SIX MINUTES AT 38°C.

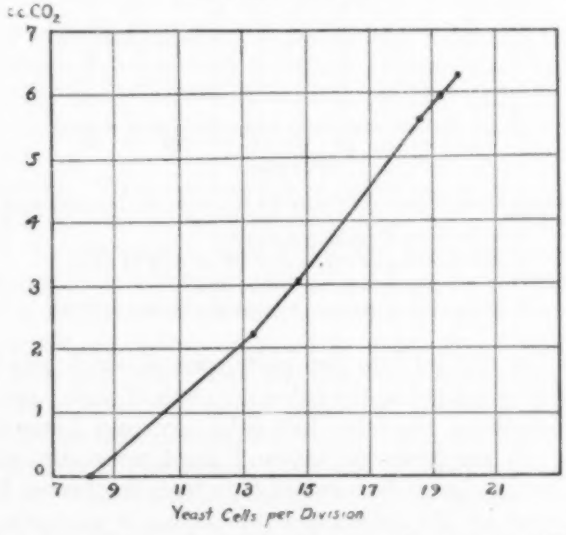

FIG. 1B. GALACTOSE FERMENTATION RATE, CUBIC CENTIMETERS CO_2 PER SIX MINUTES AT 38°C.

is whether this difference is of enzymatic nature or only due to protoplasmic-action.

We observed, that yeast, cultivated in yeast-extract with galactose, heated to 50°C., for a definite time, shows a greater decrease with respect to glucose-fermentation than with respect to galactose-fermentation.

After various orientation-experiments the following experiment was made: A quantity of yeast cultivated in yeast-extract with

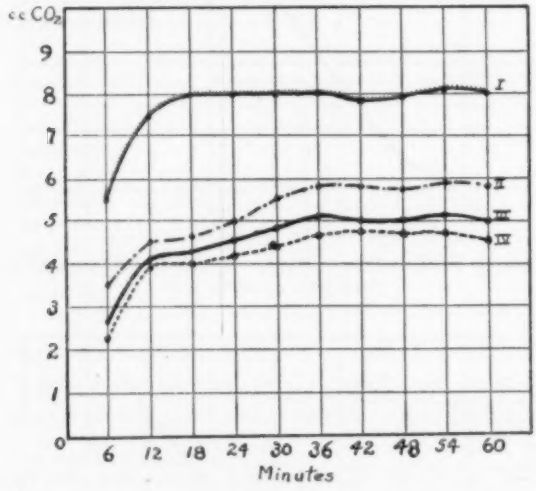

FIG. 2. YEAST CULTIVATED IN YEAST EXTRACT WITH GALACTOSE AT 38°C.

I. 6 per cent glucuse, not heated.
II. 6 per cent glucose, heated 10 minutes at 50°C.
III. 6 per cent galactose, not heated.
IV. 6 per cent galactose, heated 10 minutes at 50°C.

galactose was divided into two parts, one of which was heated to 50°C. for ten minutes, the other not; half of part 1 was given glucose to ferment, the other half galactose; part 2 was treated similarly. In graph-representation 2 the fermentation-curve is plotted; for the glucose-fermentation we see a decrease of 37.5 per cent, whereas for the galactose-fermentation it amounts to but 19 per cent. In order to observe the process more closely the

following experiment was made: 20 grams of centrifuged yeast, cultivated in yeast-extract with galactose was suspended in 500 cc. of water at 44°C. and kept in continuous motion by a stirring-apparatus. 80 cc. was removed by a pipette and the temperature of the rest quickly raised to 50°C. Half the quantity removed was transferred to 6 per cent glucose at 38°C., the other half to 6 per cent galactose and the fermentation-rates determined. After various periods quantities of 80 cc. were pipetted from the vessel at 50°C. and the fermentation-rates of glucose and galactose determined in the same way. The experiment gave the following figures:

	GLUCOSE	GALCATOSE
Original rate	15.4	9.8
After 7 minutes at 50°C	7.6	6.8
After 13 minutes at 50°C	4.7	4.4
After 20 minutes at 50°C	2.7	2.4
After 26 minutes at 50°C	2.2	2.0
After 31 minutes at 50°C	1.0	0.8

These experiments show a greater decrease of the glucose-fermentation-function than of the galactose-fermentation-function of the same yeast-cells, which indicates enzymatic differences. In graph 3 we see, that the lines do not intersect, that is, there is no galactose-fermentation after the glucose-fermentation has stopped. This corroborates the impression that we have to deal with an enzyme more resistant than the common glucose-zymase, but dependent upon it for the galactose-fermentation, that is, only converting the galactose to a product fermentable for the common glucose-zymase; in such a case the lines could not intersect, as this would point to a surplus of that enzyme and a deficiency of the common glucose-zymase at a given moment. In a later experiment there was added a considerable surplus of the common glucose-zymase, as *Sacch. Pombe*, a yeast never capable of fermenting galactose. The fermentation took place at 46°C. and the progress of the injury caused by this high temperature was indicated by the decrease of the fermentation-rates. Again we notice a quicker decrease of the glucose-

fermentation-rate than of the galactose-fermentation-rate, but of any influence exerted by the quantity of *Sacch. Pombe* there is no trace.

In a following experiment fermentation occurred at 49°C. and as an excess of common glucose-zymase exactly the same yeast was added, but cultivated in yeast-water with glucose and consequently no more capable of fermenting galactose at this temperature than the *Sacch. Pombe*.

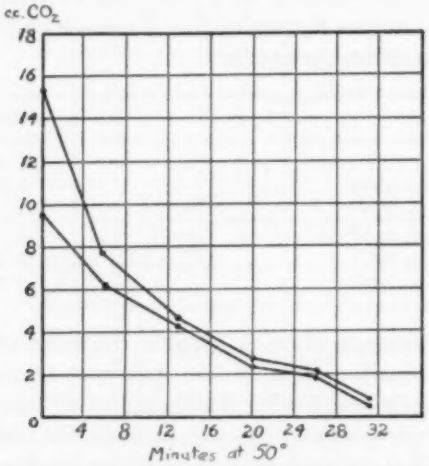

FIG. 3. CUBIC CENTIMETERS OF CO_2 AT 38°C.

Flask 1 contained yeast, cultivated in glucose-solution, fermenting glucose; flask 2 yeast, cultivated in galactose-solution, fermenting glucose; flask 3 the same yeast, fermenting galactose and in the fourth flask the same as the third with an additional equal quantity of yeast cultivated in glucose-solution. The fermentation-curves have been plotted in graph 4. No effect of the excess of common glucose-zymase is perceptible in 4. It is remarkable that here we attain a point at which the glucose-fermentation-rate has become 0, whereas the galactose is still fermented, though slowly. When this point was reached, the temperature was made to fall rapidly to 40°; the galactose in 3 and 4 persisted in fermenting for hours together, the first hour

at a rate of 10 cc. per hour, the second hour at a rate of 9 cc. per hour, while the glucose-fermentations had ceased giving a trace of CO_2. After these two hours an equal quantity of glucose was put into 3 in order to see whether it really would not cause

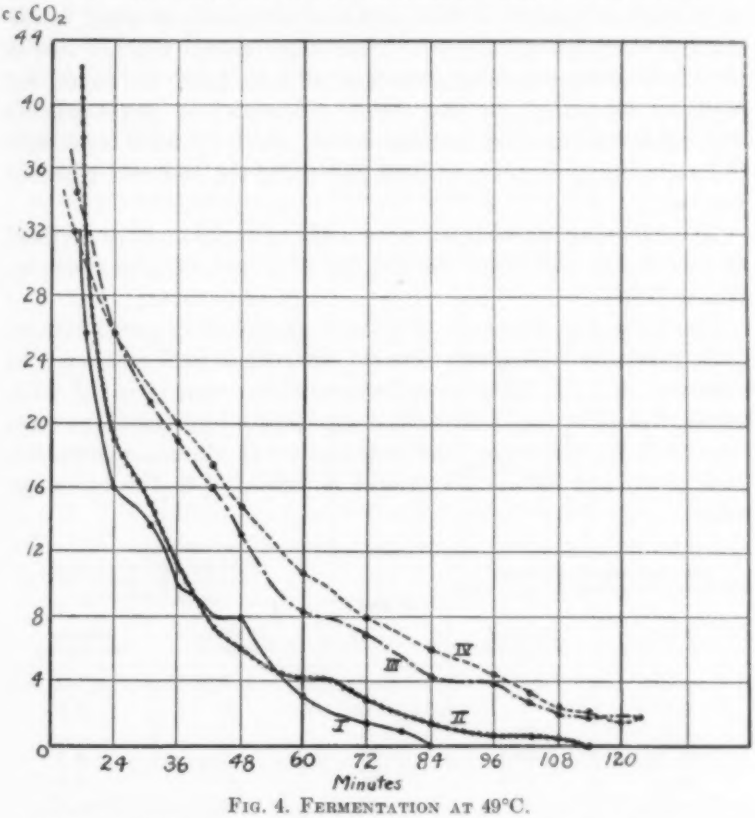

FIG. 4. FERMENTATION AT 49°C.

I. Yeast cultivated in yeast extract with glucose, fermenting glucose.
II. Yeast cultivated in yeast extract with galactose, fermenting glucose.
III. Idem, fermenting galactose.
IV. Idem plus the same quantity of yeast, cultivated in yeast extract with glucose, fermenting galactose.

any CO_2 development. The resulting quantities of CO_2 per hour were 7 cc. in 3 as well as in 4, where no glucose was added.

Before the glucose-fermentation-rate had quite decreased to 0, an inoculation on malt-agar had taken place from all 4 flasks;

none of the test-flasks gave rise to a colony on the plate; inoculations made at the beginning of the experiment served as a control. Analogous experiments gave corresponding results. So it appears possible for some fermentation to continue after the reproductive function of the yeast has completely stopped, while the galactose-fermentation function may remain slightly active after the glucose-fermentation has already been destroyed by high temperature. In the yeast, cultivated in yeast-extract with galactose, an enzyme must occur, which by itself is capable of fermenting galactose, without the aid of the common glucose-zymase.

The following experiments were made to apply another method of inhibition, dependent on the use of toluol, as was done by Giaya (1920).

One-half of a quantity of yeast cultivated in yeast-extract with galactose was given glucose, the other half galactose to ferment; half of the glucose-fermentations were treated with toluol, the other half were not; the galactose-fermentations were treated in the same way; the temperature at which fermentation took place was 23°C. The result is indicated in the following table:

6 QUARTS CENTRIFUGAL YEAST; NO TOLUOL IN THE FERMENTATION FLASKS		TIME	12 QUARTS CENTRIFUGAL YEAST; 1.5 CC. TOLUOL IN 50 CC. OF YEAST-SUSPENSION	
Glucose-fermentation	Galactose-fermentation		Galactose-fermentation	Glucose-fermentation
cc. CO_2	cc. CO_2	*minutes*	cc. CO_2	cc. CO_2
10	7	0	2	2.5
18.5	13	6		
26.5	18.5	12	7	4.5
42.5	30.5	24	9	6.5
58.5	43	36	11.5	8.5
91	67.5	60	16	11
		18	20	13.5
		30	22.5	15
		42	25	16
		54	27	17
		6	29.5	18
		18	31.5	18.5
		30	33	19.5
		42	35	21.5

When half an hour had elapsed an inoculation was made; no colony from 3 and 4 appeared on the plate; an inoculation from 1 and 2 served as control. Just as in the case of the Giaya experiments, a residual enzyme was left, after the yeast has ceased growing; we see that the residual enzyme of galactose-fermentation is larger than that of the glucose-fermentation, in spite of the fact that in the untreated flasks the glucose-fermentation proceeds more rapidly than the galactose-fermentation, as is always the case. This is in accordance with the results of the experiments with high temperatures.

Analogous experiments gave corresponding results.

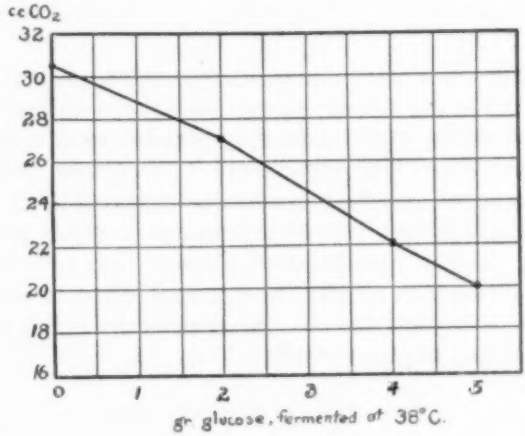

FIG. 5. GALACTOSE FERMENTATION RATE AT 38°C. CUBIC CENTIMETERS CO_2 PER SIX MINUTES

THE DISAPPEARANCE OF THE GALACTOSE-ZYMASE

Dienert ascertained that the property of fermenting galactose is lost by transferring the yeast to another sugar-solution. In order to observe the progress of this retrogression, we made the following experiments:

Of a quantity of yeast, cultivated in yeast-extract with galactose, the galactose-fermentation-rate was measured at 38°C. in the way already described (by adding 1 gram of galactose);

after the fermentation had completely stopped, glucose was added, after the fermentation of which the galactose-fermentation-rate was again determined, glucose again added, etc. The subjoined table gives the result of the experiment:

	GALACTOSE-FERMENTATION-RATE IN CUBIC CENTIMETERS CO_2 PER 6 MINUTES AT 38°C.	
	1	2
After fermentation of:		
0 gram of glucose	31	30
2 grams of glucose	28	26
4 grams of glucose	22.5	21.5
5 grams of glucose	19.5	20.5

Analogous experiments gave corresponding results. An inaccuracy appears in the increase of alcohol-concentration, which at the end of the experiment may amount to a maximum of 6 per cent. From this experiment it is evident that for the disappearance of galactose-zymase no formation of new cells is necessary, but that this disappearance proceeds almost proportionally to the quantities of glucose that are fermented. Experiments made to observe the eventual influence of the reproduction of the yeast on the disappearance of the galactose-zymase, yielded negative results.

SUMMARY OF THE RESULTS

1. The presence of *Saccharomyces cerevisiae* in a liquid nutrient containing galactose gives the newly formed cells the property of fermenting this sugar.

2. This new property consists in the appearance of a new enzyme, by itself capable of fermenting galactose.

3. The new enzyme, galactose-zymase, is more resistent than glucose-zymase, and remains under favorable conditions as a residue which is still active after the stopping of growth. By applying a high temperature it is possible to obtain a residue of the galactose-zymase separated from the glucose-zymase.

4. The disappearance of the newly formed enzyme, the galactose-zymase, is about proportional to the quantity of fermented sugar containing no galactose.

5. We may consider this phenomenon as *modification*.

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THE PENETRATION OF BACTERIA THROUGH CAPILLARY SPACES

II. MIGRATION THROUGH SAND[1]

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In the course of a series of experiments on the passage of *Vibrio percolans* (Mudd and Warren, 1923; Mudd, 1923) through Berkefeld candles, we desired to determine the motility of this organism as compared to *V. comma* (*V. cholerae*) and *Erythrobacillus prodigiosus*, which were used as controls. A modification of the method of Carnot and Garnier (1902 a) seemed the most promising procedure.

The apparatus used was a glass U-tube of 14 mm. inside diameter with arms 20 cm. long. A loose plug of glass was placed at the bottom of one arm, and a layer of 60/120 quartz sand (average diameter of grains 0.23 mm.) poured in to a depth of 10 cm. Both arms were then filled with culture medium so that the level was one centimeter above that of the sand. The arms were then plugged with cotton, and the whole autoclaved.

Each of the organisms was grown under approximately optimum conditions—hay infusion of pH 6.8 at 30°C. for *V. percolans*, nutrient bouillon of pH 6.5 at 37.5°C. for *E. prodigiosus*, and nutrient bouillon of pH 7.4 at 37.5°C. for *V. cholerae*.

SELECTION OF MOTILE STRAINS

After inoculating the layer of medium above the sand—usually done by removing 0.1 cc. of the medium and immediately

[1] Read before the Association of Pathologists and Bacteriologists, March 30, 1923.

replacing it with 0.1 cc. of a twenty-four-hour fluid culture of the organism to be tested—the tube was examined at frequent intervals, and the first perceptible clouding of the uninoculated arm was taken as the end point. This was checked by an agar plate culture. From the time required for a given organism to traverse the sand the degree of motility can be estimated.

As soon as the sand-free arm was seen to be clouded, a fresh tube was inoculated above the sand layer from the first tube. Thus the most motile organisms of each tube were selected, and used to inoculate the next tube in the series.

Inoculation was made into the small amount of medium above the sand, as it was found that when the organisms were inoculated into the large body of medium in the sand-free arm migration through the sand was very uncertain. The probable mechanism of this phenomenon will be considered below.

The strain of *E. prodigiosus* used was of very low motility, and at times was non-motile. At no time did it penetrate the 10 cm. sand layer in the fourteen days allowed to each experiment.

While different transplants of the stock cultures varied somewhat in the time of passage at the start of the experiment, the time required decreased rapidly as the organisms of higher motility—those appearing first in the sand arm—were successively selected. This increase in motility continued up to a certain approximately constant level for each of the species tested. This level of greatest motility was held, so long as transplants were made rapidly. After several generations on agar or several forty-eight hour periods of growth in fluid media, the selected strains of high motility were but little more motile than equally old culture from the stock strains.

Table 1 illustrates this selective action and also the decrease in motility due to delay in making transfers.

Three months later, after growth on a sealed agar slant, this strain of *V. percolans*, which apparently had reached a constant high degree of motility, was tested out again. It showed a rate of 0.145 cm. per hour. The rate of the stock strain at the very beginning was 0.146 cm. per hour.

In several series of experiments, both *V. cholerae* and *V. percolans* reached respectively a point of maximum motility,

TABLE 1

ORGANISM	DEPTH OF SAND	TOTAL ELAPSED TIME	RATE PER HOUR
	cm.	*hours*	*cm.*
V. cholerae	11.5	68	0.169
V. percolans 29	10.5	72	0.146
V. cholerae	10.5	43	0.244
V. percolans	10.5	55	0.191
V. cholerae	11.5	24	0.479
V. percolans	10.0	27	0.370
**V. cholerae*	11.5	27	0.426
**V. percolans*	10.5	48	0.219
**V. cholerae*	9.5	36	0.264
**V. percolans*	9.5	39	0.244
V. cholerae	10.0	24	0.417
V. percolans	10.5	28	0.375
V. cholerae	10.5	23	0.457
V. percolans	10.0	29	0.345
V. cholerae	10.5	19	0.553
V. percolans	9.5	22	0.432
V. cholerae	11.5	24	0.479
V. percolans	10.0	24	0.417
V. cholerae	11.0	20	0.550
V. percolans	10.0	24	0.417
V. cholerae	10.0	19	0.526
V. percolans	10.5	26	0.404

*Instead of inoculating a fresh U-tube directly, the organisms were grown forty-eight hours on agar, and the U-tube starred inoculated from the agar culture.

readily maintained by rapid transfers, and constant for that given strain. Carnot and Garnier (1902 b) give the maximum rates of several organisms:

	cm. per cent
B. typhosus	0.33
V. cholerae (Massouah)	0.62
V. cholerae (Paris)	0.25

The maximum of our cholera strain (Rosebank) was 0.55 cm. per hour; for *V. percolans* 0.43 cm. per hour.

Under dark field examination, the motility of the culture appeared roughly to parallel the rate of passage through the sand, though of course judgment based on a mere impression is of little value.

The method is adapted for readily raising organisms to their maximum motility, and also for separating motile from non-motile organisms. For this last purpose it is simpler and more convenient than the method of Gambier cited by Besson (1914) employing porous bougies, and the various methods using strips of filter paper.

Whether there is any increased virulence in the highly motile organisms thus selected, we have not as yet determined.

BACTERIAL CHEMOTAXIS

In the original method of Carnot and Garnier (1902 a) the bacteria to be tested were inoculated into the sand-free arm of the U-tube. In our first experiments we followed their technique in this regard. But the organisms passed through the sand layer many times more slowly than when the medium above the sand was inoculated. The bore of our U-tube was uniform—while in the tube of Carnot and Garnier, the sand-free arm was of much less diameter than the sand arm. Thus we had a considerably larger amount of medium in the sand-free arm than they did.

The sand layer forms a great number of crooked capillary tubes—the interstices between the sand grains. Diffusion through this layer is very slow. Thus 5 cc. of a 10 per cent solution of eosin placed above the sand required twenty-one days at room temperature to tinge the water in the opposite arm.

The diameter of these capillary tubes is far greater than the limiting diameter beyond which organisms will not enter such tubes—1 to 1.3 micra, as found by Hofstädter (1905). And in our own experiments the organisms passed through readily when inoculated in the small amount of medium above the sand.

The same strains of *V. cholerae* and *V. percolans* which passed the sand layer so rapidly in the experiments detailed above, when inoculated into the sand-free arm did not reach the small mount of medium above the sand, though some of the tubes were kept for twenty-three days, by which time the medium had evaporated to the level of the sand. The growth in the inoculated arm was always heavy, and the organisms showed normal motility under dark field examination. In fact, the cultures of *V. percolans* ten or more days old showed a number of small forms of even higher motility than those in the young cultures.

Since the mechanical factors were inadequate to account for our findings we sought some other explanation. While little work has been done on chemotaxis in bacteria, Pfeffer (1918) found that if *Bacterium termo* was grown in weak meat extract, an extract four times as strong was required to exert positive chemotaxis. *B. termo* and *Spirillum undula* water or saliva were attracted by 0.001 per cent pepton or meat extract.

It seemed possible that in our experiments the organisms when grown in the small amount of medium above the sand rapidly exhausted their food supply and were attracted toward the large mass of fresh medium beyond the sand. In order to check this, we set up a series of tubes, using "used" hay infusion —hay infusion in which *V. percolans* had been grown eight days, then autoclaved, and Berkefeld-filtered—instead of normal hay infusion. *V. percolans* was then inoculated by placing 2 cc. of twenty-four hour hay infusion culture above the sand layer. *V. percolans* inoculated similarly into control tubes of fresh hay infusion grew through in sixty hours. In the "used" infusion tubes the organisms did not grow through into the clear arm until the ninth day or later.

That this delay in passage was in all probability not due to inhibitory substances is shown by the following experiments.

When pepton sufficient to make a 1 per cent solution was added to the "used" infusion, the organisms grew through in ninety hours or less.

In other experiments fresh hay was soaked in the original lot of "used" infusion, the medium thus made being filtered, autoclaved, and placed in four of the tubes. When inoculated above the sand layer, the organisms grew through in eighty-four hours or less. While adsorption of some injurious substance in the "used" infusion by the added hay is not impossible, it is decidedly unlikely.

When the organisms migrate from the small amount of medium above the sand to the large amount in the clear arm, it is presumably due to their attraction up the diffusion gradient to the better food supply.

The explanation for the failure of the bacteria to reach the small amount of medium above the sand when inoculated in the clear arm would seem to be, first, that there is present in the clear arm a sufficient food supply and that no chemical gradient sufficient to cause the migration of the organisms through the sand is established from the small volume of medium above the sand to the large volume in the clear arm; and, second, that by the time the medium is exhausted, a heavy mat of dead bacteria and debris has accumulated at the lower end of the sand layer, forming a practically impenetrable barrier.

SUMMARY

A method of allowing bacteria to migrate through sand is described by which microörganisms of very high motility may be selected. Each strain of bacteria reaches a certain maximum level of activity which is retained only so long as rapid transfers are made. The method is also adaptable for separating motile and non-motile organisms. The maximum rates of migration through quartz sand attained by our organisms were, for *V. cholerae*, 0.55 cm. per hour; for *V. percolans*, 0.43 cm. per hour.

V. cholerae and *V. percolans* are shown to be subject to positive chemotaxis to nutrient media and their migration to be determined primarily by the available food supply.

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THE PENETRATION OF BACTERIA THROUGH CAPILLARY SPACES

III. TRANSPORT THROUGH BERKEFELD FILTERS BY ELECTROENDOSMOTIC STREAMING

STUART MUDD

WITH THE COÖPERATION OF

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To understand a given system it is necessary to have gained familiarity with the phenomena that occur in that system, to know what forces underlie them, and, last, and most difficult, to be able to relate these underlying forces one to the other, assigning to each its proper part in the phenomena observed. Study of the dynamics of the extraordinarily complex systems known as living organisms has made us familiar with a large array of phenomena; a number of mechanisms, too, conditioning the movements of fluids, of dissolved and suspended matter in living tissue have been brought to light. Muscular contraction with the circulation of fluids and pressure heads occasioned by it, ameboid motion of phagocytes, the beating of cilia and flagella, and protoplasmic streaming—all obviously requiring further resolution into fundamental physico-chemical forces—have been shown to play their considerable parts in the transportation of matter in living tissues. Diffusion, osmotic pressure and surface tension are known to be important kinetic forces.

It is the purpose of the present communication to consider a mechanism not so often related to the dynamics of organisms; namely, movement of liquids and suspended solids in an electric field, and to show experimentally that the forces involved

can transfer living microörganisms across a filtration membrane having certain features in common with capillary spaces found in living tissues. Whether or not such a mechanism does actually play a significant part in organisms is not under consideration in the present paper and will have to be determined by future experiment.

THEORETICAL

Two dissimilar substances, as has long been known, are seldom at the same electrical potential at their surface of contact. In biological material this interfacial potential difference is largely or wholly due to the separation of oppositely charged ions, whether by electrolytic dissociation, by adsorption in an interface (Michaelis, Freundlich and Gyemant, 1922); by unequal distribution between two phases (Beutner, 1920); or across a membrane (J. Loeb, 1922; R. F. Loeb, Atchley and Palmer, 1922; Irwin, 1922). Possibly also even at the interface of pure nonionogens, the architecture of the molecules conditions a potential difference (Frenkel, 1917).

If now there is a fall in electric potential along, i.e., tangent to, such a charged interface, the two phases will tend to be displaced with reference to each other, the positive component of the electric double layer tending to move into a region of lower, and the negative component into a region of higher, potential. In general the less firmly fixed component will be displaced with reference to the fixed component. Thus in an electric field a fluid will move through the pores of a solid membrane (electric endosmosis) and suspended particles will move through a fluid (cataphoresis).

Let us now consider a system (fig. 1) composed of a fluid with suspended particles, e.g., a culture of bacteria, bathing a diaphragm or membrane with pores of various sizes, e.g., a filter. Suppose an electric field to be set up across the filter. According to the Helmholtz-Smoluchowski equations (1914) the fluid next the pore walls will stream toward the pole of opposite sign with a velocity.

$$V_{en} = \frac{F\zeta_s . K . H .}{4\pi . \eta} \qquad (1)$$

All units are C.G.S. H is the electric field strength in E. S. U. per cm. η is the coefficient of viscosity of the medium. K is the dielectric constant of the medium.[1]

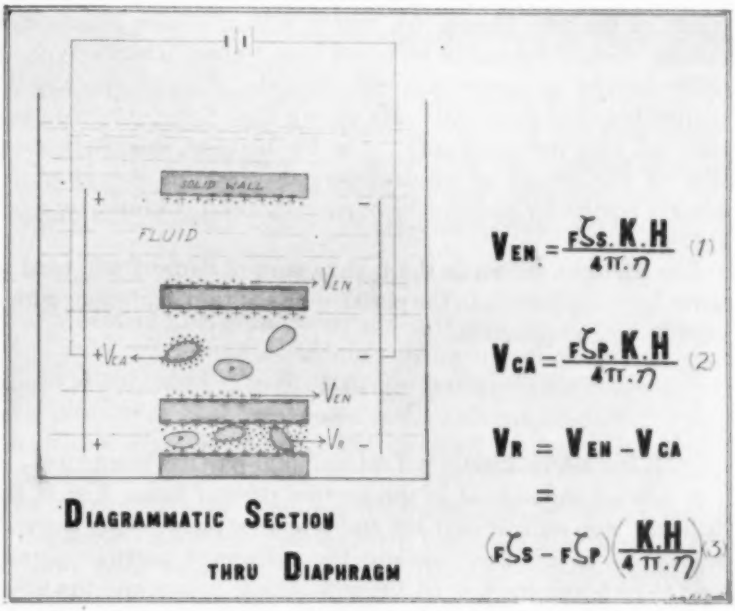

FIG. 1. SCHEMATIC REPRESENTATION OF ELECTROCAPILLARY EFFECTS

In uppermost pore of diaphragm an endosmotic current moves toward the cathode with a velocity V_{en}. In middle pore suspended particles move toward the anode with a velocity of cataphoresis, V_{ca}. In bottom pore the two effects are combined, with a resultant movement of the particles toward the cathode at an approximate velocity, $V_R = V_{en} - V_{ca}$.

$F\zeta s$ is the electrokinetic p.d. between fluid and pore walls, $(\varphi i - \varphi a)$ of the Helmholtz equations, and requires a further word of explanation.

[1] This has practically always been taken as equal to the constant of the pure medium, regardless of the solute it contains, thus involving a serious uncertainty (Lattey, 1921, Keller, 1921, Errera, 1922).

The layer of water molecules next the wall is conceived in the Helmholtz theory as held immovably fixed to the wall. The displacement in an electric field takes place between this fixed fluid layer and the body of the fluid, between which there is the potential difference ζ. This ζ-potential Freundlich (1922) therefore calls the electrokinetic potential. The total p.d. between the inside of the two phases, (ϵ), which with oil-water phases containing electrolytes can be reckoned from Nernst's thermodynamically derived concentration cell formula, Freundlich calls the thermodynamic potential. He shows that the ζ- and ϵ-potentials can vary independently. ζ is, for instance, extremely sensitive to the effects of electrolytes adsorbed in the interface. Similar results for proteins have recently been obtained by Loeb (1923).

The particles shown in the middle pore of figure 1 will tend to move by cataphoresis to the positive side of the diaphragm with a velocity of cataphoresis.

$$V_{ca} = \frac{{}_F\zeta_P . K.H.}{4\pi.\eta} \qquad (2)$$

${}_F\zeta_P$ is the electrokinetic p.d. at the fluid-particle boundary.

If now we suppose, as in the bottom pore of figure 1, that the capillary is so narrow that the endosmotic streams along the wall occupy its entire cross section[2] the suspended particles should tend to be borne one way by the endosmotic stream and the other way by cataphoresis. Combining the two equations we get the resultant velocity,

$$V_R = V_{en} - V_{ca} = ({}_F\zeta_S - {}_F\zeta_P)\frac{K.H.}{4\pi.\eta} \qquad (3)$$

It is evident that if the particles were at a higher potential than the medium the velocity of cataphoresis would be added to that of endosmosis (v. footnote 3), and similar changes in

[2] The observations of Cotton and Mouton, (1906) suggest that glass capillaries of diameter less than 50 μ at any rate would fulfill this condition. Quartz or gypsum capillaries would have to be narrower than those of glass (1906).

sign would have to be made if the direction of the potential gradients indicated by ${}_F\zeta_S$ or H were reversed, as might well happen in a living system.

The value of V_R so derived must be regarded as qualitatively only and not quantitatively correct, since the homogeneous fall in potential along the capillary walls, ${}_F\zeta_S$, would probably be interfered with by the presence of the particles with their fields (Smoluchowski, 1914, paragraph 19).

It is also to be emphasized that the value of V_R so derived only applies to a membrane on the two sides of which the fluid pressure is and remains the same. If the ends of the capillary spaces of the membrane are closed or are at different pressures, counter currents would develop within the capillaries (Ellis, 1911).

EXPERIMENTAL

The object of the present experiments was to drive electrically a culture medium containing living bacteria through an artificial membrane. They illustrate the mechanism set forth above, and are in qualitative agreement with equation (3). The experimental arrangement was not adapted to exact quantitative treatment.

The apparatus used is shown diagrammatically in figure 2. The diaphragm is a diatomaceous earth Berkefeld filter, such as is commonly used in bacteriology for obtaining sterile filtrates. It is of the most porous or "V" type. The filter is severed from its metal mountings to prevent short circuiting, and is sealed with gold size varnish into a rubber stopper which is itself cemented with gold size into one end of a glass cylinder. In the other end of the cylinder is a second stopper fitted with a carbon electrode and four glass tubes; three open into the cylindrical vessel and are used, respectively, for introducing buffer solution into the vessel, for equalizing pressure within and without the vessel when it is inverted for withdrawal of the filtrate, and for pouring off the filtrate. The fourth tube has a platinum wire sealed into its closed tip, which contains mercury, and with a similar tube inside, serves for estimation of the p.d. acoss the filter. The inoculation

tube at its outer end is thrust through a rubber collar inside the neck of a funnel, thus facilitating sterile inoculation of the filtrate into vessels of sterile culture medium.

The cultures used for the experiments were mixed growths in hay infusion of *Erythrobacillus prodigiosus* and *Vibrio percolans*. The former is the conventional organism used in experiments with filter-passing organisms to prove the tightness of the

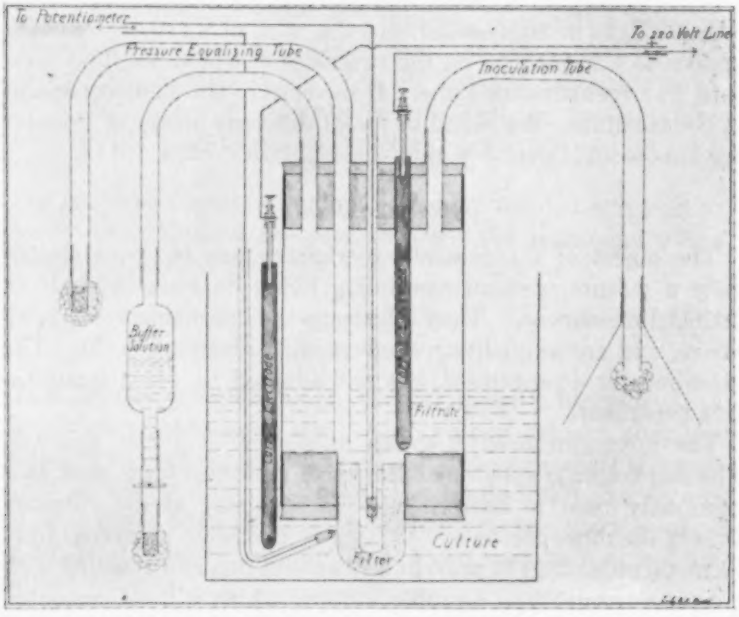

FIG. 2. SECTION THROUGH APPARATUS USED FOR ELECTROENDOSMOTIC TRANSPORT OF V. PERCOLANS (SLIGHTLY SCHEMATIC)

Filtration vessel sterile and protected from outside contamination

filter for ordinary bacteria. *V. percolans* is a non-pathogenic, motile vibrio isolated by one of us, which, under appropriate conditions, passes regularly through Berkefeld candles of the "V" type (1923).

The buffer solutions were phosphates colored by brom-thymol-blue. In the earlier experiments mixtures of the primary and

secondary phosphates were used; later the pure dihydrogen phosphate was added to the inner vessel fluid to neutralize the effects of the alkali there formed by electrolysis.

The filter as diagrammed in figure 2 is proportionately larger than in the actual apparatus. Its walls are about 4 mm. thick and are traversed by irregular and tortuous spaces narrowing down to minimal diameters of about 0.4μ (4×10^{-5} cm.). A length of filter varying in different experiments from 1 to 4 cm. was exposed below the stopper.

The potential source was the laboratory 220 volts d.c. line. The drop in potential across the filter wall was irregularly distributed over its surface, and varied widely according to position of electrodes and distribution of electrolyte through the system. The limits in the most intense part of the field were about 10 to 70 volts. This p.d. had to be measured with a potentiometer; voltmeters, because of the high resistance of the solutions, gave too low readings. The current strength varied from 0.02 to 0.1 ampère. The rate of filtration (i.e., electroendosmotic transport) under the above circumstances was about 2 or 3 cc. per minute.

The experimental procedure was as follows:

The apparatus was assembled as described. Cotton was adjusted to protect the exposed ends of all tubes and the funnel of the inoculation tube was covered with a towel. The whole was autoclaved, a Diack control being used to insure sterilization. When cool, the lower end of the filtration vessel and filter were soaked in distilled water until the liquid level inside was just high enough to cover the tip of the carbon electrode. A few cubic centimeters of M/25 NaH_2PO_4 solution containing bromthymol-blue were allowed to run into the filtration vessel. In the later experiments, i.e., with three exceptions from experiment E_{18} through E_{36}, the amount necessary to neutralize the alkali produced by electrolysis was added to the filtration vessel before the closing of the circuit and no further additions were made.

A suspension of *V. percolans* and *E. prodigiosus* was prepared either, in the early experiments, by suspending surface agar growths in dilute culture medium, or later (E_{15} through E_{36}), by

using diluted or undiluted hay infusion cultures strained to remove flocculi and surface pellicle. The filter was then immersed in the suspension up to the inner fluid level. The circuit was closed. As the inside fluid level slowly rose the filtration vessel was lowered to keep inside and outside levels approximately the same. The circuit was broken, the filtration vessel removed from the culture and the filtrate was inoculated into vessels containing sterile hay infusion. Inoculation was made in a chemical "hood" whose metal floor was the top of an electrically heated steam bath. By previously washing the air within this chamber with steam, contaminations were reduced to a minimum. Finally one tube was inoculated as a control with the unfiltered culture. All inoculated vessels were kept in an incubator at about 30°C. If the filtrate contained vibrios one or more of the inoculated vessels showed clouding after twenty-four to sixty hours. The control tube, inoculated usually with 3 loops of unfiltered culture, regularly showed clouding in twenty-four hours due to a mixed growth of the two organisms, and often of others introduced by air contamination of the open outer vessel.

The organisms in the clouded vessels were routinely identified by examination with the dark field illuminator, by Gram-stained preparations and by plating out on agar. Vessels in which no clouding appeared after a week were designated negative(−), (table 1). Vessels in which *V. percolans* was identified in pure culture were called positive (+). The control tubes were called positive (+), when a mixed growth of *V. percolans* and *E. prodigiosus* was demonstrated in them.

The results are given in table 1. During the early experiments results were somewhat variable, chiefly because of imperfect technique. Later it may be seen that, with the exception of an occasional negative experiment, vibrios were quite regularly grown in pure culture from the filtrates of runs of six and one-half minutes or longer. About 15 cc. of fluid were filtered in this time. Experiments of less than six and one-half minutes duration were negative.

Though it is perhaps sufficiently evident that the vibrios were not passing by their own unaided efforts from the nutrient

TABLE 1

EXPERIMENT NUMBER	FILTER NUMBER	NUMBER OF TIMES FILTER USED PREVIOUSLY	DURATION OF EXPERIMENT IN	NUMBER OF VESSELS POSITIVE	NUMBER OF VESSELS NEGATIVE	CONTROL TUBE
			minutes			
E1	14 or 15	0	30	Leak about stopper; all tubes mixed growth		
E2	14 or 15	0 or 1	36	1?	3	+
E3	14 or 15	1?	40	1?	4	+
E4*	17?	0	30	1?	2	+
E5	17	1?	31	7	1	+
E6	11	0	32½	0	11	+
E7	15	2?	26	0	8	+
E8	14	2?	15	0	9	+
E9	17	2?	28	0	8	+
E10	11	1	36	5	0	+
E11	18	0	27	1	8	+
E12	16	0		3	5	+
E13	20	0	22	2	0	+
E14	21	0	25	3	4	+
E15	22	0	30	7	4	+
E16	23	0	25	0	8	+
E17	24	1 (for control)		8	0	+
E18	25	1 (for control)	25?	0	9	+
E19	26	1 (control)	38	3	1	+
E20	24	1 control 1 experiment	25	3	1	+
E21	27	1 control	28	9	0	+
E22	27	1 control 1 experiment	30½	5	0	+
E23	26	1 control 1 experiment	15	2	0	+
E24	16	1 control 1 experiment	8½	0	3	+
E25	11	1 control 2 experiments	10+	2	0	+
E26	22	1 control 1 experiment	10	2	1	+
E27	17	1 control 3 experiments	11½	1	1	+
E28	21	1 control 1 experiment	9½	2	0	+
E29	25	1 control 1 experiment	10	0	3	

*The apparent negative results of the first experiments were due partly to the fact that the free alkali in the filtration vessel was not neutralized by the addition of buffer. From Experiment E5 on, buffer was added in all experiments.

TABLE 1—*Continued*

EXPERIMENT NUMBER	FILTER NUMBER	NUMBER OF TIMES FILTER USED PREVIOUSLY	DURATION OF EXPERIMENT IN	NUMBER OF VESSELS POSITIVE	NUMBER OF VESSELS NEGATIVE	CONTROL TUBE
			minutes			
E30	28	1 control	9	2	0	+
E31	29	1 control	9½	2	0	+
E32	29	1 control 1 experiment	6½	0	2	+
E33	28	1 control 1 experiment	8½	2	0	+
E34	30	1 control	7	1	1	+
E35	32	1 control	$5\frac{7}{6}$	0	1	+
E36	33	1 control	6	0	2	+
E37	31	1 control	6½	1	1	+

medium outside the filter into acidified distilled water within it, nevertheless a series of control experiments were run to obtain more definite evidence on this point. For these the apparatus and culture were prepared exactly as for an experiment except that the circuit was not closed. The fluid levels inside and out naturally remained unaltered and the vibrios had to pass, if at all, by their own motility from culture medium without to distilled water within the filter.

In old filters, i.e., filters in whose pores nutrient substances remained from previous filtrations, vibrios were cultivated from the fluid within the filtration vessel after exposures of nineteen hours and some minutes, of ninety minutes, and of seventy-seven minutes. Negative results were obtained with exposure of old filters for ninety-two and ninety minutes. With new filters no positive fluids resulted from exposures of less than two hours (table 2.)

Behavior of the motile vibrios in an electric field has been observed a number of times in a simple cataphoresis chamber made by conducting current through electrolyte-soaked cotton threads to an ordinary blood counting chamber of the Levy single field type. Endosmotic streaming may be observed in the fluid immediately adjacent to the top and bottom of such a chamber and cataphoresis in its more central parts. No evidence of orientation of the motile organisms in the electric field has been observed, however.

It is thus evident that the vibrios are borne passively through the filter by the endosmotic stream as they would be by streaming produced by gravity or other source of energy. In this case, however, the inward movement of the organisms in the endosmotic stream is partially opposed by the outward movement by cataphoresis of the organisms through the stream, so that they are not so rapidly conveyed into the filtrate as they would be by streaming at a similar rate produced by gravity. This is

TABLE 2

CONTROL EXPERIMENT NUMBER	FILTER NUMBER	NUMBER OF TIMES FILTER USED PREVIOUSLY	DURATION OF EXPERIMENT	NUMBER VESSELS POSITIVE	NUMBER VESSELS NEGATIVE	CONTROL TUBE
I	17	2 or 3	1 hour 30 minutes	0	3	+
II	20	1	19 hours 20 minutes	3	0	+
III	22	1	1 hour 32 minutes	0	3	+
IV	21	1	19 hours 50 minutes	2	0	+
V	25	0	1 hour 38 minutes	0	2	+
VI	24	0	1 hour 43 minutes	0	1	+
VII	26	0	1 hour 3 minutes	0	3	+
VIII	27	0	2 hours 43 minutes	3	0	+
IX	11	2	1 hour 30 minutes	3	0	+
X	16	1	1 hour 17 minutes	2	0	+
XI	28	0	2 hours (approx.)	1	1	+
XII	29	0	2 hours 10 minutes	1	3	+
XIII	30	0	1 hour 31 minutes	0	2	+
XIV	31	0	1 hour 45 minutes	0	2	+
XV	32	0	1 hour 30 minutes	0	2	+
XVI	33	0	1 hour 45 minutes	0	3	+

illustrated by comparison of the results in table 1 above, with tables 1, 2, and 3, of a previous communication on filtration of *V. percolans* (Mudd, 1923). Rates of streaming of the same order were produced by the electric field in one case and by suction in the other. With suction filtrates were uniformly inoculated with vibrios (when normal) in two to six minutes; by endomosis at least six and one-half minutes were required.

Had the vibrios been positively instead of negatively charged their velocity of cataphoresis would have been added to the endosmotic current instead of subtracted from it, as is evident from equation (3).

It will be noted that the potentials used in the above experiments were high. Forty volts, for instance, over a path of 8 mm. through the filter wall, would be equal in intensity to a potential difference of 200 millivolts over a path of 40μ through a membrane. This is a larger p.d. than is to be anticipated in a living system. The pressures developed by the endosmotic current under the conditions of the experiments were correspondingly high, 60 to 220 cm. of water (measured by rise of filtrate in a manometer tube projecting from the filtration vessel). Moreover as previously shown (1923), the mechanical difficulties of the vibrios passing the filter at all are great. We were breaking new ground and used forces much larger than we should expect under the conditions of nature.

DISCUSSION

The experiments reported above were undertaken under the stimulus of certain observations regarding passage of bacteria and other particles through the mucous membranes of the tonsils. A brief résumé of these observations may serve as an example of a concrete medical problem in the further investigation of which it is hoped the mechanism outlined above may possibly serve as a guide.

Jonathan Wright in 1896 (1896) figured tubercle bacilli passing through the intact laryngeal epithelium of a patient with tuberculous laryngitis. Wood (1914) later gave hogs tuberculosis and anthrax by swabbing cultures of the specific organisms lightly on the animals' tonsils, and demonstrated the anthrax bacilli penetrating between the cells of the tonsillar epithelium. The crypt bacteria were kept out until the epithelium had been devitalized by the invading anthrax organisms. J. L. Goodale (1897) in 1897 dusted carmine on the surface of tonsils shortly before excision and on subsequent microscopic examination, found the carmine particles passing in between the tonsillar epithelial cells and in the direction of the efferent lymph channels. The indigenous bacteria of the crypts, though of about the same size as the carmine granules, could only rarely be found in or beneath the epithelium. He believed that the carmine was

carried in by a stream of buccal fluids resorbed through the tonsillar epithelium into the cervical lymph vessels. M. Hendelsohn (1898) reported similar experiments with several types of particles penetrating both the faucial and pharyngeal tonsils. He reported the granules as being both intercellular and intracellular as they passed the epithelium. Jonathan Wright subsequently confirmed and extended these observations; the foreign particulate matter was taken in between the cells, the cryptal bacteria were kept out. A. S. Warthin (1923) has recently confirmed both the selective transfer through the tonsillar epithelium and the intercellular course of the particles admitted.

Wright (1909) has especially urged the importance of seeking physico-chemical mechanisms capable of explaining such selective action by the first line of defense against infectious disease. What forces act at membranes and are capable of importing foreign particles and excluding the crypt bacteria?

One naturally thinks of osmotic pressure. It is hard to understand a *selective* influence of osmotic pressure. Surface tension has been especially mentioned by Wright. That surface tension can transport bacteria along phase boundaries in a striking manner has been shown by us elsewhere. The electrocapillary mechanism described would also seem for the present at least a possible working hypothesis. The question as to whether or not it will be found to play a significant part in the phenomena under consideration will have to wait upon further investigation.

Another possible application of the electrocapillary mechanism developed above may be worth mentioning here. An electrical disturbance is known, from the studies of Bayliss and Bradford (1885), of Cannon and Cattell (1916), and of Gesell (1919), to result from stimulation of glands. This electric effect is strikingly correlated with the outflow of secretion from the glands, and is not necessarily correlated with changes in blood supply. Bradford (1887), by ligating the efferent ducts of the glands did not significantly alter the electric variation, and thus showed that the flow of secretion along the gland duct was not the cause of the disturbance in potential. The converse possibility, namely that an electric p.d. between the ends of the cells bordering the lumen

and the peripheral ends next the blood supply might cause the outflow of secretion by a mechanism analogous to that developed above, does not seem to have been considered in the literature. It seems to have sufficient possibility at least to be worth testing.

An alternative possibility in secretion is that stimulation of the gland may cause an increased permeability to osmotically active cell components of that part of the cell membrane bordering the lumen (Bayliss, 1920). In that case, as Bayliss has pointed out, osmotic pressure would cause a streaming of water through the cells into the lumen. On the other hand, such an osmotic mechanism does not seem adequate in such a case as resorption in the kidney tubules where water is passed from tubules into blood stream against osmotic pressure. The possibility that electroendosmotic streaming is here involved is at least to be considered.

Be it understood: this paper does not, and does not aim to, prove anything in regard to what actually happens in the organism in infection, resorption, secretion or other process. It is simply an exposition of a mechanism, one among many, by which fluid and suspended particles may be moved through capillary spaces, and as such it may or may not turn out to have a significant part in vital processes. It is perhaps of interest, first because overlooked by most biologists, and, second, because it is *selective* in nature.[3] Should future study reveal suitable potential gradients, (expressed as H in equation 3 above), and evaluate the charges between particles and their environing medium, $({}_F\zeta_P)$, and between the walls of intercellular spaces and their contained

[3] As an example of its selectivity suppose the following circumstances:

The walls of the capillary spaces are charged negatively to -30 millivolts against the fluid in them, $({}_F\zeta_a = -30)$. Suppose one type of particle to be charged also to -30 millivolts, $({}_F\zeta_p = -30)$. Suppose a second type of particle to be charged positively to 20 millivolts, $({}_F\zeta'_p = +20)$. From equation (3) we have $V_a = (-30 + 30)\frac{K.H}{4\pi\eta} = 0$, and $V'_a = (-30 - 20)\frac{K.H}{4\pi\eta} = -50\frac{K.H}{4\pi\eta}$. Thus the first particles would undergo little or no movement, whereas the second particles would move toward the cathode at a velocity about $\frac{5}{7}$ that of the endosmotic stream. It is known that the charges of cells vary according to their constitution and to the hydrogen and other ion concentrations of the fluids bathing them, (Coulter; Northrup and DeKruif; Eggerth and Bellows, 1921–22).

fluids ($_F\zeta_S$), it is hoped that the relations described by the above equations may at least show how the electrokinetic effects would tend to move the fluids and particles. Whether the actual movement would be approximately that given by V_R in any case would of course depend on whether electrokinetic effects alone were operative, or were combined with movements due to osmotic pressure, gravity, muscular contraction, etc.

SUMMARY

Experiments are reported in which a filterable microörganism, *Vibrio percolans*, was drawn through artificial membranes by electroendosmotic streaming. The membranes were Berkefeld "V" filters. Their wall thickness was about 0.4 cm. Their pores were irregular and tortuous, narrowing down to minimal diameters of about 0.4μ, (0.4 + 10^{-4} cm.)

The difference in potential across the membranes varied from ten to seventy volts. The source was carbon electrodes connected to the laboratory 220 volt, direct current, line. The endosmotic transport of fluid across the filter under these circumstances was 2 to 3 cc. per minute. The pressures developed were those of 60 to 220 cm. of water.

Vibrio percolans is negatively charged relative to its medium, and thus tends to move by cataphoresis to the anode. Water streams by electroendosmosis through the negative filter pores to the cathode. The resultant movement of the microorganisms under the combined influence of cataphoresis and endosmosis is in this case with the endosmotic stream to the cathode. Appearance of vibrios in the filtrate about the cathode is much quicker than when they penetrate the filter by their own motility alone and somewhat slower than when they are filtered by suction. These results are in qualitative agreement at least with the equations ofthe Helmholtz-Smoluchowski theory set forth above.

Possible applications of this mechanism of electroendosmosis and cataphoretic movement are indicated in relation to penetration of bacteria through epithelia and in relation to secretion and resorption.

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A DESCRIPTION OF A SELF-VENTILATING CONSTANT TEMPERATURE INCUBATOR ROOM

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The incubator rooms in common use for bacteriological purposes are open to objections in regard to constancy of temperature at a given level or at different levels, and also in regard to the occurrence of dead air spaces or heat pockets in the various parts of the room. These objections can only be obviated by making use of a well insulated room, an efficient thermoregulator and heating units, together with a mechanism which insures continual circulation of the air. In my experience the insulation of the room, the thermoregulator and the electrical heating units present little difficulty; the adaptation of a proper ventilation system suitable for bacteriological work is more of a problem. Generally, fans are resorted to for the purpose of stirring the air. This procedure is not only disadvantageous in that contaminations are apt to occur in the vicinity of the fan—this is especially true with petri dish cultures—but also because of the nuisance experienced in taking care of the motor to assure constant operation day and night. An attempt was accordingly made to secure circulation of the air by making use of the well known laws of thermo-circulation. A room constructed with this idea in mind has been quite satisfactory, and because of this fact a brief description will be given.

ROOM

The inside dimensions of the room are 8 feet wide, 8 feet high and 12 feet long. Insulation is obtained by means of a 4-inch wall, the outside of which consists of the ordinary walls of a

room made of terra cotta tiles and cement: the inside lining is a composition fire-proof board about $\frac{3}{8}$ inch thick. This board is fastened in place to studding (2 by 4) set about 16 inches apart. The space between the walls of the room and the lining of the incubator room is filled with cork dust and asbestos wool. The bottom of the incubator is the ordinary hard wood floor laid on a cement base. The door, a plain wooden paneled pattern, reinforced on one side by a layer of this fire-proof board, leads into the room from an anteroom, 8 by 9 by 6, thus providing in effect a double door. The fresh air is supplied from the anteroom by means of adjustable slits in the top and bottom of the door.

CIRCULATION OF AIR

To insure circulation of the air, baffle boards were placed on two sides of the room 7 inches from the wall with the heating units between the baffle boards and the walls of the room; thus, in effect, a flue was formed in the space between the baffle boards and the walls of the room. The baffle boards are made of the same fire-proof board mentioned above and are held in place by being bolted to iron brackets. A schematic drawing showing the position of the baffles is given in figure 1. The baffles were placed 1 foot from the floor and extended to within 2 feet of the ceiling and 7 inches of the side walls. The heating units consisted of a very efficient commercial heater made in units 2 feet in length, each unit consuming 250 watt hours of electricity. These were fastened by means of iron brackets 4 inches from the wall of the incubator, 3 inches from the baffle board and 18 inches from the floor. They extend the full length of the sides of the room. In operation the warm air rises, taking the direction indicated by the arrow in figure 1. The temperature at the top of the baffle is on the average 40°C. and at the bottom, 4 to 6 inches from the floor, 34° to 35°C. The baffle board serves another purpose, in that it acts as additional insulation for the room, the air between it and the wall being warmer than that of the rest of the room. It is thus conceivable that the insulation of the wall need not necessarily be as efficient as the one used in this room; an air space between the outside and the inside wall being perhaps sufficient.

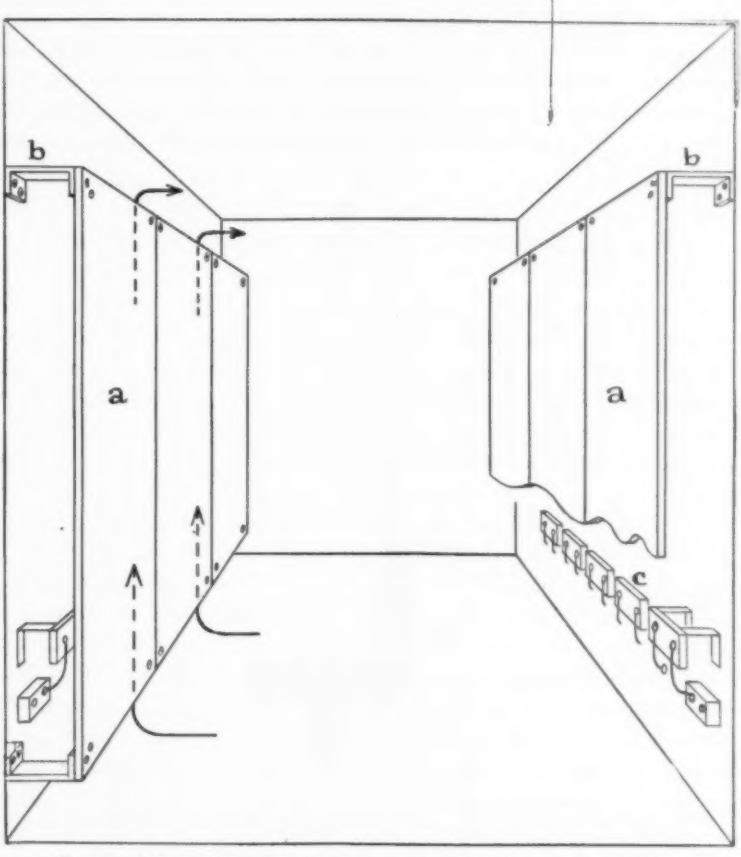

FIG. 1. A SCHEMATIC REPRESENTATION OF THE INCUBATOR ROOM
a = Baffle boards.
b = Iron brackets to which baffle board is fastened.
c = Heating units.

THERMOREGULATOR

During the last eight years the author has made use of many types of thermoregulators, commercial and "home-made." Experience has shown that for water-bath or incubator room, no regulator approaches in constancy a well constructed mercury thermoregulator. One commercial bi-metallic type, used for over two years with constant oversight day and night, refused

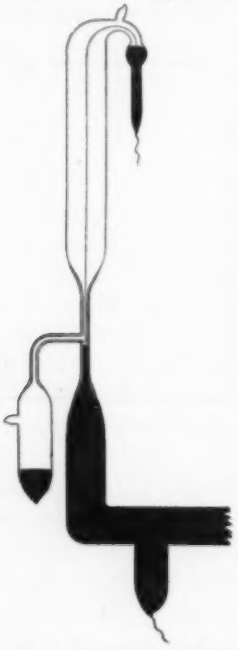

FIG. 2. A REPRESENTATION OF THE HEAD OF THE MERCURY THERMOREGULATOR

to operate as many as five times during that period, destroying the results of months of work: this happened despite the fact that repairs were made to obviate the difficulty. The regulator used here is one described by Clark (1) with a slight modification of the head. Inasmuch as some difficulty has been experienced in maintaining a constant position of the contact wire over a long period of time, because of the lack of rigid fixation to the

adjustment screw, and for the reason that the sealed type of head designed by Clark did not permit of an easy adjustment of the mercury level, a head for the Clark mercury grid was devised as represented in figure 2. At the restricted portion of the head, just above the contact position, a capillary tube with a reservoir for mercury is attached so that mercury can be transferred from one reservoir to the other. Changing the level of the mercury is accomplished readily. If the level is too high, the mercury in the grid is warmed slightly so that it rises above the side arm capillary to a desired amount, the upper part of the head is then heated, and the pressure caused by the warmed gas forces the mercury through the capillary tube into the side arm reservoir. If the mercury is too low, the regulator, turned so that a little mercury fills the capillary from the side arm reservoir, is brought to a vertical position and the tube heated to drive the small amount of mercury into the regulator proper. In this manner, very small quantities of mercury can be transferred, allowing a regulation of at least 0.1°C. Nicrome wire was used for making contact and was sealed, first in pyrex tubing, and then to insure a tight seal a bit of shellac was melted over the glass seal.

No attempt will be made to describe in detail the electrical connections as they are of the usual laboratory type. The regulator is operated from a divided 110 volt circuit, so that 4 volts at approximately 1–1000 ampere activate a master relay which in turn controls a commercial relay capable of breaking a 40-ampere current. In actual operation this relay breaks about 7 amperes. The major supply of heat for the room comes from 4 constant units which are sufficient to raise and keep the temperature at about 34°C., but not above. This constant unit arrangement has also the advantage of maintaining a constant circulation of air, which would not occur if all heating units were operated by the relay, shutting off and on the entire current.

TEST OF TEMPERATURE REGULATION

Two methods were used to test the constancy of temperatures: the one, by means of a recording thermometer sensitive to about 0.1°C. placed in position about midway between ceiling and floor,

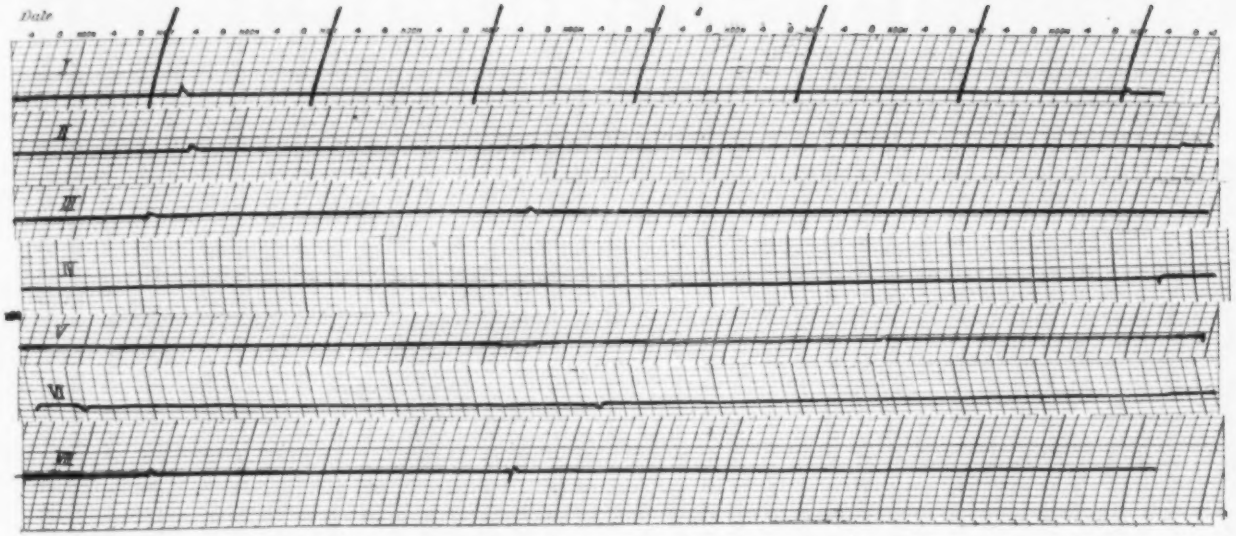

FIG. 3. WEEKLY CHARTS MADE BY A RECORDING THERMOMETER

I. Week of 1/3/23
II. Week of 1/8/23
III. Week of 1/16/23
IV. Week of 1/24/23
V. Week of 2/1/23
VI. Week of 2/8/23

Tracings were reinforced by black ink

and the other by 18 thermometers fixed in many different positions on a rack $2\frac{1}{2}$ by 6 by 18 feet set in the middle portion of the room. The results shown by the recording thermometer over a 7-week period are given in figure 3. In all these records, divergence from the straight line was explained by a local heating

TABLE 1

Temperature at different locations in the room

	DATE			
	November 22, 1922	November 31, 1922	December 9, 1922	December 17, 1922
Position I:				
Top	37.8	37.8	37.8	37.6
Middle	37.3	37.5	37.5	37.5
Bottom	36	36.2	36.4	36.1
Position II:				
Top	38	38	38	38
Middle	37.3	37.6	37.6	37.6
Bottom	36.5	36.7	36.7	36.6
Position III:				
Top	38	38	38	37.8
Middle	37.6	37.6	37.6	37.6
Bottom	37	37.1	37.2	37
Position IV:				
Top	38	38	38	38
Middle	37.6	37.6	37.6	37.6
Bottom	37	37.2	37	37
Position V:				
Top	38	38	38	37.8
Middle	37.8	37.7	37.7	37.7
Bottom	37.1	37.2	36.8	36.8
Position VI:				
Top	38	38	38.1	38.1
Middle	37.4	37.6	37.6	37.6
Bottom	35	35.5	35.7	35.2

Readings were made at about 11.00 a.m. on the different days.

effect caused by the use of a Bunsen burner which was employed in the room and near the regulator to carry over sterile transfers. The fluctuation of the temperature at times when the heaters are on and when they are off is scarcely noticeable: that there is a slight variation is apparent from the breadth of the mark made by the recording needle; in fact, by the aid of a hand lens small

waves in the tracing may be observed. However, inasmuch as the recording thermometer is so sensitive that 0.1°C. variation in temperature results in a millimeter swing of the recording needle, the fluctuations in temperature lie between 0.1° and 0°C.

Table 1 discloses the variation in temperature in different parts of the room. The thermometers used were of ordinary chemical types registering from 0° to 110°C.; the 18 used, picked from a large number, did not vary more than 0.1°C. when exposed to a temperature of 37.5°C. It is at once evident that the temperature at the same level is quite constant, the top being the warmest and the bottom the coolest. Position VI (table 1) at the bottom has always been a cool pocket. This pocket is at the end of the room opposite the door where circulation of air is restricted by shelving and other apparatus. The average difference between top and bottom, however, is small compared to that found in three other incubator rooms used by the author, the smallest variation in these rooms being 2.5° and the largest .5°C. Furthermore, the fluctuations in temperatures at the various levels in the other rooms referred to were inconstant, the bottom being the hottest when the heating units were operating. It is my opinion that by use of the principle here described even better circulation of air can be obtained, making possible an incubator room of ideally constant temperature, provided greater care is observed in construction of the baffle, both in regard to the size of the air space between the baffle board and the walls (to permit of more rapid circulation of the air) and also to the structure of the baffle, so that radiation effects may be negligible.

SUMMARY

An incubator room is described in which circulation of air is maintained by means of the heating mechanism. The heating units are placed in a flue which consists of a space between the baffle boards and the walls of the incubator room. Constant air currents are thus obtained by means of thermo-circulation. The difference in temperature between the top and bottom in a 5-foot space averaged about 1.5°C., but at any one level in

the room, it is ideally constant. A modification of the head for the Clark mercury grid thermoregulator is described by which it is possible easily to make adjustment of the mercury level after the outlets of the head are permanently sealed.

REFERENCE

(1) Clark, William Mansfield: The Determination of H-ions. Williams & Wilkins, 1922.

COAGULATION AND STERILIZATION OF LOEFFLER'S MEDIUM IN THE AUTOCLAVE

MARTIN DUPRAY

Hutchinson, Kansas

Received for publication, August 27, 1923

From time to time there have been efforts to avoid the tedious inspissation and fractional sterilization of Loeffler's blood serum culture medium. Van Saun and Graves[1] have recently described a method for preparing this medium in Petri dishes. They coagulated the serum by heating it in the autoclave four or five hours with the door three-fourths closed; then the door was wholly closed and sterilization accomplished by heating for twenty minutes at 15 lbs. pressure. Hinkleman[2] has described a much shorter method for preparing Loeffler's medium, in tubes in which the tubes are tightly corked and placed in a special container with a lid that can be tightly clamped down on the corks to keep them from blowing out. The container is placed in the autoclave with the tubes in the proper slanting position and the pressure is raised to 10 pounds; the heat is then turned off and the autoclave allowed to cool down. Hinkleman attributes the absence of bubbles or shrinking to his method of preventing expansion in the medium during the heating. As the heating in his process is insufficient for complete sterilization of material with much contamination, he uses fresh serum handled aseptically, with occasional failure of sterilization even then.

These notes have prompted me to describe a method that I have used for the past nine years with success. The procedure is carried out in the ordinary autoclave without special apparatus, is complete in half a day, and gives a sterile medium even with

[1] Van Saun, Anna I., and Graves, Isabelle A., Jour. Lab. & Clin. Med., 1923, **8**, 406.

[2] Hinkleman, A. J., Jour. Bact., 1923, **8**, 315.

badly infected material. I worked this method out at the University of Illinois in 1912 but neglected to publish it at that time. Since then I have taught it to a number of students and introduced it into three other laboratories. In addition I believe it has been in use in several other laboratories for some time. It is published now because I do not believe it has yet appeared in print or that it has become common knowledge.

Beef blood serum is used that has been collected without any precautions to avoid contamination; 2 ounces of chloroform are added to each gallon of serum. The serum is stored in the ice box and used as needed. It is often six or eight months before the supply is used up. No spoilage takes place, but the serum is not sterile. It is necessary to remove the chloroform before the serum is used. To do this, the amount of serum needed is measured out, placed in an aluminum or granite ware cooker with handle, warmed to about 40°C., and with another similar cooker at hand is slowly poured from one to the other until the odor of chloroform is gone. This takes three to five minutes.

The serum-broth mixture is then prepared as usual, tubed, and the tubes plugged with cotton. The tubes are placed in the autoclave in the proper slanting position. The door of the autoclave is then closed, and care should be taken that the seating is perfect and that there are no leaks. The air escape valve is *closed*. Heat is then applied (or steam turned on if connected to live steam) and the pressure brought gradually to 5 pounds. The cold air in the autoclave is *not* allowed to escape. The pressure is maintained at 5 pounds for two hours. The temperature of the cold air trapped in the autoclave rises gradually and the medium heats up slowly enough to coagulate without bubbling or shrinking. At the end of two hours, increase the pressure gradually to 10 pounds, at the same time opening the air escape valve slightly until steam appears, then closing it again. Run at 10 pounds pressure for twenty to thirty minutes. Turn off heat and allow pressure to come down to zero. It is essential that the cooling should not be rapid. If the pressure inside the autoclave comes down faster than the temperature of

the medium, boiling will occur and the medium be ruined. This let-down of pressure faster than the temperature of contents happens occasionally with an autoclave in a cold room; it happens often if the autoclave leaks steam. As a precaution I have found it advisable to turn off the heat gradually (or the steam, if connected to live steam).

The resulting medium is white, has a smooth surface and is sterile. The method has been used repeatedly with success on Loeffler's medium in Petri dishes. Fifteen pounds pressure may be used for final sterilization if desired. The higher pressure tends to make the surface of the medium a little friable and easily broken by loop or swab, but does not appear to injure it in any other way.

It is essential that the air escape valve be closed during the first stage of the heating, and that the autoclave shall not leak, otherwise the cold air escapes and the interior of the machine heats up too fast. The success of the method depends on trapping the cold air in the autoclave. I believe the success of Hinkleman's method depends as much on the heat insulating action of the air in his stoppered tubes as on the prevention of expansion.

A STUDY OF Á BACTERIAL ASSOCIATION

I. THE BIOCHEMISTRY OF THE PRODUCTION OF LACTIC ACID

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Department of Zymology, University of Toronto

Received for publication September 22, 1923

During the late war large amounts of acetone and butyl alcohol were manufactured in various countries by the fermentation of cereals and other carbohydrates. It was to be expected that the industrial application of such a process would involve numerous difficulties, including those in connection with sterility and the preservation of pure cultures. One of the many interesting observations of scientific interest made during this period was the fact that the majority of unsatisfactory fermentations due to contamination in England, the United States and Canada were essentially alike in their biochemical and bacteriological characteristics (Nathan, 1919, Reilly and others, 1920, Thaysen, 1921, Speakman, 1920). In addition to the anaerobic bacillus responsible for the production of acetone and butyl alcohol the mash in these fermentations invariably contained a small bacterium, which in stained preparations could be seen in the form of short chains (plate I, fig. 1). This organism gave rise to small, circular colonies on aërobic plates or slopes of lactose nutrient agar (plate I, fig. 2). It has been described in greater detail by Thaysen (1921). At the end of twenty-four hours growth in artificial medium the organism contains numerous large volutin granules, which stain pink in a preparation lightly stained with methylene blue. Lactic acid is formed in cultures containing glucose, maltose or lactose. There is little if any, amyloclastic activity in media containing starch. The organism does not form spores. It was regarded by Thaysen as a new species, and named *Bacterium volutans*.

[1] Bursar of the Honorary and Advisory Research Council, Ottawa.

The biochemical characteristics of the contaminated fermenters were as follows: After a brisk evolution of gas lasting for about 20 hours after the time of inoculation with the culture of *B. granulobacter-pectinovorum*, the rate of gas production, instead of continuing to rise, fell rapidly to zero. During this period, and for many hours after gas production had ceased, the acidity of the mash steadily increased to very abnormal figures. Reilly and co-workers (1920) have shown that this mash is very rich in lactic acid. The yields of acetone and butyl alcohol from such a fermentation were sometimes nil. In view of the fact that the contaminant is a producer of lactic acid, the first explanation we entertained of these phenomena was the simple and obvious one, namely, that contamination had overcome by more rapid growth the butyl alcohol organism, and utilized any soluble carbohydrate in the medium to form lactic acid. Any toxic action exerted by *B. volutans* we assumed to be due to the general and specific action of the high acidity produced. These conclusions had to be abandoned as soon as we isolated pure cultures of *B. volutans*, and investigated the behavior of this organism growing alone and in association with *B. granulobacter-pectinovorum* in different media. We shall endeavour to show in this communication that the lactic acid produced in mixed cultures is not produced by *B. volutans* but by *B. granulobacter-pectinovorum*, and that this change in the metabolism of the latter organism is due to the action of some metabolic product of *B. volutans* which inhibits partially or completely the majority of its normal biochemical processes.

In the recent literature reports have been made of three bacterial associations similar to the one we are investigating. The association containing *B. coli* and *B. paratyphosus* has been carefully investigated by Smith and Smith (1920–21). They show that *B. coli* produces no gas but the normal amount of acid in lactose broth, which has been previously inoculated with *B. paratyphosus*. The factor which is responsible for the inhibition of gas production increases in power as the time allowed between the two inoculations is increased up to four days. After remaining stationary for several days it gradually loses strength, and

disappears from the medium at the end of three or four weeks. By separating the cells of the *B. paratyphosus* culture from their metabolic products, and submitting these two components to heat and other treatment, the authors have endeavored to determine the nature of the inhibitory agent. They regard the available experimental evidence as inconclusive, but offer the tentative hypothesis that it is some metabolic product of *B. paratyphosus*. Owing to the fact that it is destroyed at temperatures slightly above the thermal death point of the bacteria they conclude that it is possibly an enzyme.

The organism responsible for the characteristic flavor and "eyes" of a Swiss cheese is *B. acidi-propionici*. It has been shown by Sherman and Shaw (1920–21) that the amount of propionic acid produced by this organism in an artificial medium containing lactose is increased six or seven-fold by adding to the same medium a culture of *Streptococcus lacticus* or *Lactobacillus casei*. The possibility of the increased yield of acid being due to the formation of propionic acid from the lactic acid produced by the associated organism has been eliminated by the fact that similar results were obtained by the addition of organisms which do not ferment lactose. The authors did not investigate further the biochemistry of the association.

The third case is discussed in a paper by Fouassier (1921) which we have only been able to review in abstract form. Brief mention is made of the fact, that the production of lactic acid by an organism isolated from milk is stimulated by association with *B. subtilis* and Tyrothrix.

EXPERIMENTAL

Cultures. The cultures of *B. granulobacter-pectinovorum* used in this investigation were derived from the laboratory stock culture. Those of *B. volutans* were originally derived from pure cultures of the organism isolated in 1917 from a typical contaminated fermenter. We found that these cultures, on continued growth in artificial media, lost their toxic properties and so we were compelled to adopt a method for the isolation of a fresh toxic strain.

Three 300 cc. Erlenmeyer flasks containing 200 cc. of 3 per cent maize mash are sterilized in the usual manner. To each flask 5 to 10 grams of unsterilized maize meal is added, and they are then inoculated with 1 or 2 cc. of a vigorous culture of *B. granulobacter-pectinovorum*. The flasks are incubated at 36°C. At this stage they contain the varied flora of the meal in addition to the culture added. The latter soon begins to ferment and produces CO_2 and H_2. The result is that only obligate or facultative anaerobes can possibly develop in the medium, and usually at the end of three or four days the mash contains only *B. granulobacter-pectinovorum* and *B. volutans*. Strains of *B. mesentericus* occasionally persist in small numbers. A 10 cc. sample of the mash in each flask is then titrated with 0.1 N NaOH in order to discover whether the characteristic high acidity of a mixed culture has been formed. *B. volutans* is isolated from the acid mash by the usual bacteriological methods, and cultivated in wort or yeast water.

Experiment I. Three experimental flasks containing 750 cc. of 3 per cent maize mash were sterilized for two hours at 15 pounds steam pressure. They were allowed to cool to room temperature, and inoculated in the following manner with pure cultures of *B. granulobacter-pectinovorum* and *B. volutans:*

Flask A: Inoculated with 10 cc. of *B. granulobacter-pectinovorum.*

Flask B: Inoculated with 10 cc. of *B. granulobacter-pectinovorum* 10 cc. of *B. volutans.*

Flask C: Inoculated with 10 cc. of *B. volutans.*

The *B. volutans* culture used was derived from a contaminated fermenter. The flasks were incubated at 36°C., and periodic determinations of the acidity of 10 cc. samples were made. The pure culture of *B. granulobacter-pectinovorum* alone gave a normal fermentation. In flask B there was a vigorous fermentation for about thirty hours, and then gas production diminished very rapidly. There was a considerable residue of unfermented starch. There was no sign of gas production or starch hydrolysis in flask C. The results from the experiment are given in table 1 and figure 2.

The results from the experiment indicated that it was possible to reproduce in the laboratory under controlled conditions the

TABLE 1

TIME AFTER INOCULATION	ACIDITY*		
	A	B	C
hours	cc.	cc.	cc.
4	0.2	0.2	0.2
12	1.2	1.0	0.3
22	3.5	3.1	0.7
25	3.4	3.6	
29	2.9	4.1	0.9
34	2.0	4.4	0.9
48	1.8	7.1	1.0
58	1.7	8.5	1.1
72		9.4	1.1
97	2.0	9.6	1.2

* The titratable acidity in this and other tables is expressed in terms of 0.1 N acid per 10 cc. of medium.

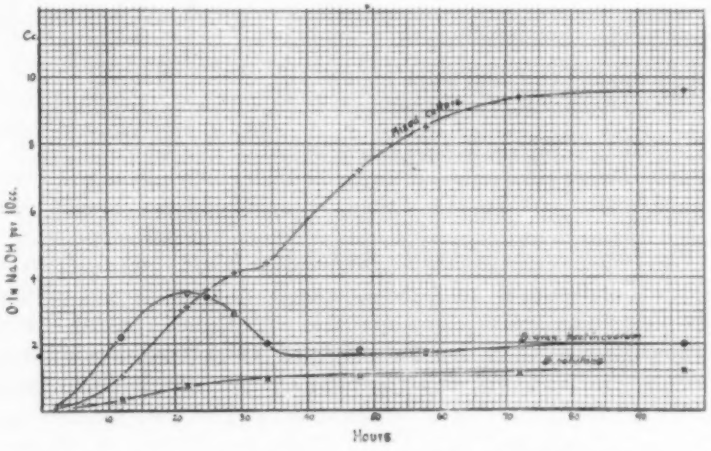

FIG. 1. CURVES SHOWING THE ACIDITIES OF PURE CULTURE FERMENTATIONS BY BOTH ORGANISMS AND OF A MIXED-CULTURE FERMENTATION

contaminated fermentation process of the plants. Much to our surprise we found that the *B. volutans* culture alone did not produce a large amount of acid in maize mash. Still considering that in a mixed culture the lactic acid was produced by this organism, and not by *B. granulobacter-pectinovorum*, we sought

for a possible explanation of the non-production of high yields of lactic acid in flask C. The most logical explanation seemed to be that it was owing to the inability of *B. volutans* to hydrolyse starch. In the mixed culture this operation would be performed by the glucoamylase secreted by *B. granulobacter-pectinovorum*. We therefore studied the behavior of pure cultures of *B. volutans* in media containing lactose or glucose.

Experiment II. Experimental flasks containing different media were sterilized, inoculated and incubated. Samples were withdrawn at intervals from the flasks, and titrated. The information regarding media and inoculum used, and the experimental results are summarized in table 2.

TABLE 2

FLASK	MEDIUM	INOCULATION		ACIDITY AFTER			
		B.v.	B.g.p.	16 hours	37.5 hours	43 hours	60 hours
		cc.	cc.	cc.	cc.	cc.	cc.
A	Broth + 1 per cent maize meal + 2 per cent lactose....	10	0	0.5	0.9	1.0	1.1
B	Same.....	1	10	1.5	3.2	3.5	4.0
C	Broth + peptone + 3 per cent lactose.....	10	0	0.8	1.1	1.1	1.3
D	Same.....	1	10	1.6	2.6	2.8	3.0

We found that in media containing maize and lactose, or peptone and lactose, pure cultures of *B. volutans* do not produce acidities which are at all comparable with those of mixed-culture fermentations. The results obtained from media containing glucose also indicated that the failure to obtain high acidities in maize-mash cultures of *B. volutans* was not due primarily to a shortage of glucose.

Owing to the fact that we still considered *B. volutans* to be the main producer of lactic acid in the contaminated fermentation we carried out experiments to discover whether the products of the *B. granulobacter-pectinovorum* fermentation are used up to form lactic acid. These products would be absent from pure cultures of *B. volutans*, and the low acid production under these circumstances could then be explained. Flasks of maize mash

were fermented to different degrees by *B. granulobacter-pectinovorum*, and then sterilized without loss of volatile products. They were then inoculated with pure cultures of *B. volutans*. The results gave us no encouragement to believe that this was the solution of our problem. We were thus compelled to adopt the hypothesis that the lactic acid formed in a mixed culture was mainly due to the activity of *B. granulobacter-pectinovorum*, and only indirectly to the presence of *B. volutans*. At this time it was known that in pure cultures of *B. granulobacter-pectinovorum* there is a small amount of non-volatile acid, and work was in progress to determine the nature of this acid. In a recent paper (1923) we have shown that in pure cultures of *B. granulobacter-pectinovorum* small amounts of lactic acid are produced. Having adopted the hypothesis that in mixed cultures this particular process is activated in some unknown manner we proceeded to carry out the following experiments.

Experiment III. Four experimental flasks each containing 750 cc. of 5 per cent maize mash were sterilized and inoculated with varying amounts of the two cultures.

Flask A: received 6 cc. of *B. granulobacter-pectinovorum*.

Flask B: received 6 cc. of *B. granulobacter-pectinovorum* + 0.75 cc. of *B. volutans*.

Flask C: received 6 cc. of *B. granulobacter-pectinovorum* + 1.5 cc. of *B. volutans*.

Flask D: received 6 cc. of *B. granulobacter-pectinovorum* + 3.0 cc. of *B. volutans*.

The flasks were incubated together, and acidity determinations made in the usual manner. All the flasks containing a mixed inoculum gave typical acid fermentations. On two occasions the mash in flask D was analyzed by the Duclaux method for volatile and non-volatile acids. The results from the experiment are given in table 3. The curves in figure 2 are based on the results from the control flask A and flask D.

The results given in the first part of table 3 brought to our attention a most suggestive fact, namely, that the highest acidity was not obtained in flask D, which received the largest amount of *B. volutans* culture, but in flask C, which received only one-half of this amount. This matter was investigated further in experiment IV.

TABLE 3

TIME AFTER INOCULATION	ACIDITY			
	Flask A	Flask B	Flask C	Flask D
hours	*cc.*	*cc.*	*cc.*	*cc.*
18.5	3.95	3.20	2.90	3.20
22.0	3.90	4.20	3.80	3.90
29.5	2.40	4.50	4.80	5.20
42.5	3.00		7.30	7.00
80.0	2.90	7.70	9.60	8.70

Duclaux analysis of Flask D

TIME AFTER INOCULATION	ACIDITY DUE TO		
	Acetic acid	Butyric acid	Lactic acid
hours	*cc.*	*cc.*	*cc.*
29.75	0.90	1.50	2.75
80.00	0.85	1.45	6.27

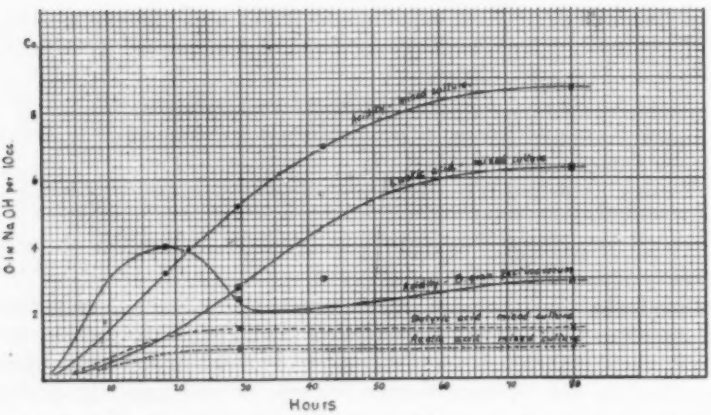

FIG. 2. CURVES SHOWING THE ACIDITY OF A CONTROL, FLASK A, AND OF THE MIXED CULTURE IN FLASK D; ALSO THE DEVELOPMENT OF ACETIC, BUTYRIC AND LACTIC ACIDS IN FLASK D

The results obtained from the Duclaux analyses provide an interesting study when compared with similar results from a fermentation by a pure culture of *B. granulobacter-pectinovorum* (Speakman, 1923, fig. 1). During the first eighteen hours the con-

taminated fermentation differed in the following respects from the normal. There was a reduced production of butyric acid and acetic acid, but an increased production of lactic acid. The most striking differences developed during the period from the twentieth hour to the end of the fermentation. In the contaminated medium gas production fell off rapidly to zero. There was no further production or utilization of volatile acid and consequently no further production of neutral substances. The production of lactic acid continued at an increased rate for about 40 hours, and then gradually fell to zero. The final concentration of lactic acid in the medium was very high, amounting to 6.27 cc. of 0.1N acid per 10 cc. of mash. This amount is considerably higher than that obtained by the cultivation of *B. volutans* in media containing maize, glucose or lactose. It is also greater than the sum of the amounts obtained in separate pure cultures of the two organisms in any of these media. The results obtained indicate that the greater part of the lactic acid was produced by some abnormal physiological type of *B. granulobacter-pectinovorum*. This being the case we considered that there ought to be some definite relationship between the growth of *B. granulobacter-pectinovorum* and the amount of lactic acid produced. Similarly if, by increasing the dosage of *B. volutans* cultures, we could progressively inhibit the growth of *B. granulobacter-pectinovorum*, the yields of lactic acid should vary inversely with the amounts of added *B. volutans* culture. To test the validity of this hypothesis the following experiment was carried out.

Experiment IV. Five flasks of 3 per cent maize mash were sterilized, cooled and inoculated. Each flask received the same volume of *B. granulobacter-pectinovorum* culture, but four of the flasks received different volumes of the *B. volutans* culture. Acidity determinations were made at regular intervals, and the results from the experiment are given in table 4.

The results from this experiment show that as we increased the volume of *B. volutans* in the inoculum, the final acidity of the mash fell. In similar experiments we were able, by further increasing the amount of *B. volutans*, to inhibit to a greater degree

the normal fermentation, and reduce the amount of lactic acid produced. These experiments as a whole afforded additional evidence in favor of our hypothesis that the lactic acid formed is largely produced by *B. granulobacter-pectinovorum*.

Experiment V. We endeavored in this experiment to determine in a more quantitative manner the relationship between the amount of growth and normal activity of *B. granulobacter-pectinovorum*, and the degree to which lactic acid is produced in mixed cultures. The first of these two measurements was made by determining the loss in weight due to gas production. The second measurement was made by titration. Erlenmeyer flasks containing 200 cc. of 3 per cent maize mash

TABLE 4

Effect of increasing quantities of B. volutans in inoculum

FLASK	INOCULUM		ACIDITY OF FLASK AFTER					
	B.g.p.	B.v.	7 hours	12 hours	24.5 hours	31 hours	48 hours	72 hours
	cc.	cc.	cc.	cc.	cc.	cc.	cc.	cc.
A	10	0	1.0	2.3	3.6	2.8	2.3	2.4
B	10	1	1.0	1.7	5.0	5.8	7.4	7.7
C	10	2	0.8	1.5	4.3	5.2	6.0	7.0
D	10	3	1.1	1.5	4.1	5.3	6.7	7.4
E	10	5	0.8	1.2	2.7	4.1	5.8	6.6

were sterilized, and cooled. They were then inoculated with varying ratios of the two cultures. Each flask was than closed with a sterilized rubber stopper fitted with an Alwood valve containing H_2SO_4. Before incubation the series of flasks was carefully weighed. At the end of three days they were removed from the incubator and weighed a second time. A 10 cc. sample from each flask was titrated. The results from this experiment are given in table 5 and figure 3.

The results indicate that the fermentations in this group were of three types. Flasks A to D gave normal fermentations, although Flask C was slower than the remainder for some unknown reason. The amounts of *B. volutans* culture added to these flasks had no apparent effect on the metabolism of *B. granulobacter-pectinovorum*. Flasks D to F were all acid fermentations in which only a relatively small amount of carbohydrate was

TABLE 5

Effect of increasing quantities of B. volutans in inoculum

FLASK	INOCULAM		LOSS IN WEIGHT	ACIDITY
	B.g.p.	B.v.		
	cc.	*cc.*	*gm*	*cc.*
A	2.0	0.0	3.605	3.0
B	2.0	0.025	4.069	3.5
C	2.0	0.075	2.422	3.3
D	2.0	0.15	3.711	3.15
E	2.0	0.25	0.461	6.0
F	2.0	0.50	0.454	5.8
G	2.0	1.00	0.387	4.8
H	2.0	3.00	0.183	2.7
I	2.0	5.00	0.205	2.9

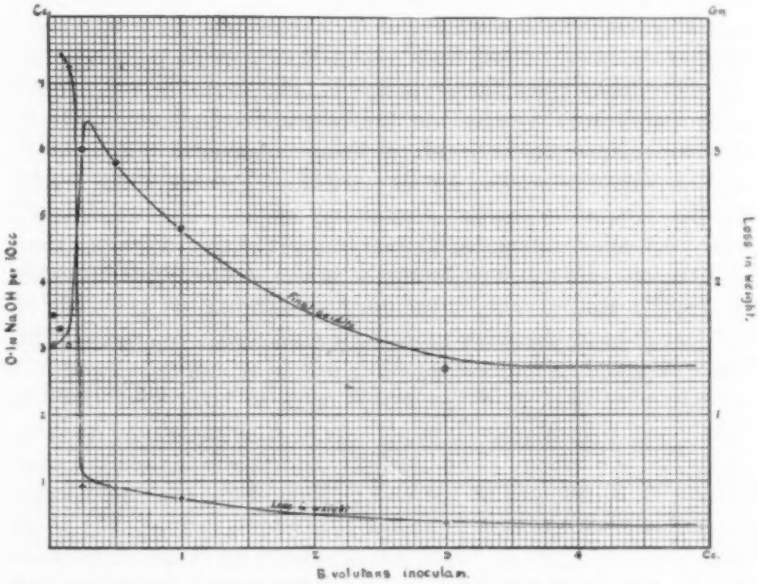

FIG. 3. CURVES SHOWING THE INFLUENCE OF INCREASING AMOUNTS OF *B. volutans* ON THE DEGREE OF NORMAL FERMENTATION BY *B. granulobacter-pectinovorum* AND ON THE FINAL ACIDITY OF THE MEDIUM

utilized. The titration figures show that there is a definite relationship in this group of flasks between, (a) the activity of *B. granulobacter-pectinovorum* as measured by gas production, (b) the amount of *B. volutans* culture added, and (c) the amount of lactic acid produced. The amount of lactic acid produced varies directly with (a) and inversely with (b). These results confirm those obtained in experiment IV. In Flasks H and I there was only a meagre production of gas, and the final acidities were also low. This experiment as a whole was repeated, and the results obtained confirm those which we have given.

DISCUSSION

The results from the experiments which we record show quite clearly that what we regarded during the war-period as a typical contaminated fermentation was only one of several possibilities resulting from the association of these two bacteria. There are fermentations containing both organisms which are indistinguishable chemically from the normal. This type can be reproduced in the laboratory by the addition of small volumes of toxic *B. volutans* culture to the mash, or large volumes of non-toxic *B. volutans* culture obtained by the continued growth of the organism in laboratory media containing sugar. The amount of inhibitory substance added to the mash at the time of inoculation, plus the amount which is developed in the medium, is insufficient to disturb the normal biochemical equilibrium of the cells of *B. granulobacter-pectinovorum*. A second type, represented by flasks H and I in experiment V, is one in which the amount of inhibitory substance in the inoculum is so large that there is hardly any normal growth or activity of *B. granulobacter-pectinovorum* in the mash. The medium contains practically a pure culture of *B. volutans* at the end of three days, and the normal amount of lactic acid is produced by this organism. The third and most interesting type is represented by flasks B to E in experiment IV and flasks E to G in experiment V. The amount of toxic material in the original *B. volutans* culture added to these flasks is not sufficient completely to inhibit the growth and activity of *B. granulobacter-pectinovorum* during the first twenty

hours of the normal fermentation period. Partial inhibition, proportional to the volume of *B. volutans* culture added, does occur however. The result of this relationship is that we find a sub-normal production of gas and volatile acid during this period. At this point, which marks normally the beginning of the most active part of the fermentation, the concentration of the toxic material in the original *B. volutans* inoculum, or more probably this amount plus whatever has been developed in the mash, is sufficient to inhibit completely the following biochemical processes which normally are active in *B. granulobacter-pectinovorum:* (a) the production of volatile acids, (b) the oxidation and reduction of volatile acids, and (c) the oxidation of lactic acid. By each of these processes gas is also produced, and it is possible to follow the progress of the inhibition by observing the rate of gas production. The only normal process which continues in the cells of *B. granulobacter-pectinovorum* is the production of lactic acid. If we interpret the biochemistry of the association in this manner we observe that it resembles the association of *B. coli* and *B. paratyphosus* very closely. There is the same fundamental principle involved, namely, the partial inhibition of one organism by one or more of the metabolic products of the second.

Apart from the experimental evidence contained in this paper for the existence of a type of *B. granulobacter-pectinovorum* which is abnormal in the physiological sense, descriptions exist in the literature of similar mutants from closely allied species. The following passage is taken from the report of Bredemann's exhaustive study of the amylobacter group (1909, p. 446).

> These observations, that at the same time it is possible to transform a strictly anaerobic spore-forming bacillus, which is a vigorous fermenter, into an aerobic, non-spore forming and non-fermenting coccus, seemed so surprising that I have convinced myself of the correctness of this phenomenon with quite unexceptionally pure cultures. The microoidia could not only be observed on one stem, but all stems in so far as I have tested them showed these phenomena in exactly the same manner.

To show the importance of this passage in connection with our problem it is necessary to observe that Bredemann would include *B. granulobacter-pectinovorum* in the amylobacter group. Similar conclusions were recorded in an earlier paper by Winogradsky (1902) who found that it was possible to develop a form of *Clostridium pasteurianum* which differed from the parent stock both morphologically and physiologically. The morphological aspects of this most interesting problem have been discussed recently in great detail by Löhnis (1921). Concerning ourselves only with its physiological aspects, we find that the micro-oidia are produced by heat treatment, or by the accumulation of acid products in the medium owing to the absence of $CaCO_3$. They produce no gas in media containing carbohydrate, but continue to produce acid. It has been shown by Grassberger and Schattenfroh (1907) that from bacilli which normally produce butyric acid, a new bacterium occasionally develops which produces no gas, and although acid production continues, the acid formed is lactic instead of butyric. The literature which we have cited concerning these physiological mutants, confirms our conclusion that in the association which we are investigating, the rapid production of lactic acid is due to a disturbed equilibrium in the physiological processes of *B. granulobacter-pectinovorum*, the change in equilibrium being brought about by the inhibition of certain biochemical processes by some inhibitory factor in the *B. volutans* culture. We are unable to state definitely whether our physiological mutant corresponds morphologically to the micro-oidia of Bredemann. At no stage however in our investigation have we been able to obtain aërobic growth corresponding to that described in the literature.

During the course of our experimental work we have been able to make some observations which are suggestive as to the nature of the inhibitory factor in the *B. volutans* culture. From the original culture of this organism we have obtained a strain which grows luxuriantly in laboratory media with the production of lactic acid, but these cultures do not inhibit or change the physiological behavior of *B. granulobacter-pectinovorum*. It would seem improbable therefore, that the cells of *B. volutans*

or the products of carbohydrate metabolism are the inhibitory agent. We have also found that the production of this factor in cultures of *B. volutans* is dependent upon, (a) a meagre supply of soluble carbohydrate, and (b) a supply of vegetable protein. We have adopted therefore the tentative hypothesis, that the inhibitory agent is a product of the nitrogen metabolism of *B. volutans*.

SUMMARY

1. The biochemistry of the association *B. granulobacter-pectinovorum-B. volutans* has been qualitatively and quanitatively investigated.

2. It has been shown that the characteristic production of large amounts of lactic acid by this association, in media containing carbohydrate, is due to a partial inhibition of the physiological processes of *B. granulobacter-pectinovorum* by some factor produced in *B. volutans* cultures.

3. The inhibitory factor is not characteristic of cultures of *B. volutans* grown for several generations in artificial media containing sugars.

4. The tentative hypothesis has been adopted, that this substance is a product of the nitrogen metabolism of *B. volutans* growing in media containing vegetable protein and only traces of carbohydrate material.

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PLATE 1

FIG. 1. Preparation made with mash from a contaminated fermenter, showing *B. granulobacter-pectinovorum* in the form of faintly stained rods and the more deeply stained chains of *B. volutans*.

FIG. 2. Colonies of *B. volutans* on an aerobic wort-agar plate. The stroke-culture was made from the same mash as the slide of figure 1.

FIG. 3. Preparation made from a pure culture of *B. volutans* in wort. The culture was derived from a single colony fished from the growth in figure 2.

FIG. 1

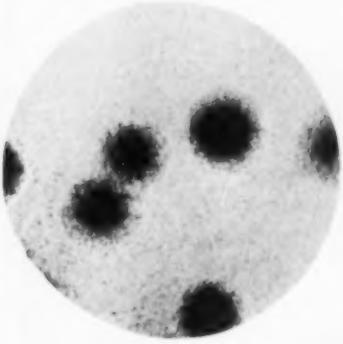

FIG. 2

FIG. 3

(Speakman and Phillips: Biochemistry of a bacterial association)